Ludwig Maximilians University, Munich
Faculty of Biology
Zoological Institute

Max Planck Institute of Psychiatry
Neuroendocrinology Group

Study on transcription factors involved in the pathogenesis of pituitary adenomas

Dissertation

Submitted on 17. December 2001 by
Marily Theodoropoulou

1. Berichterstatter: Prof. Dr. Rainer Landgraf
2. Berichterstatter: Prof. Dr. Peter Schlegel

Tag der mündlichen Prüfung: 2. Oktober 2002

Contents

INTRODUCTION

The pituitary gland

The pituitary gland, or hypophysis, is a major control point for the proper function of the endocrine system. It has a small size (average weight in human normal male adult: 0.6gr), and it resides within a midline depression of the sphenoid bone, the sella turcica (Fig.1). It is composed of two lobes, the anterior pituitary/ adenohypophysis, and the posterior pituitary/ neurohypophysis (Scheithauer et al., 1996). The pituitary gland develops from two embryologically different parts: an invagination of the oral ectodermal, known as Rathke's pouch; and the *infundibulum*, a downward extension of the diencephalon. The cells of the anterior wall of Rathke's pouch differentiate and rapidly proliferate under the influence of certain transcription factors to form the adenohypophysis (Fig.2; Kioussi et al., 1999; Sheng et al., 1996), while the posterior wall gives rise to the pars intermedia. The *infundibulum* gives rise to the pituitary stalk and to the neurohypophysis.

The neurohypophysis is composed of modified glial cells, the pituicytes, and nerve fibers, extending from the hypothalamus, with their nerve endings. The adenohypophysis is composed of the *pars distalis*, which is the largest part of the gland containing the hormone producing cells; the *pars intermedia*, filled with microcysts - rudiments of the Rathke's pouch; and the *pars tuberalis*/*infundibularis*, which is an upward extension of the anterior lobe towards and around the pituitary stalk. Although *pars intermedia* is a prominent and functionally significant feature in the rodent pituitary, in humans it seems to have little significance.

i

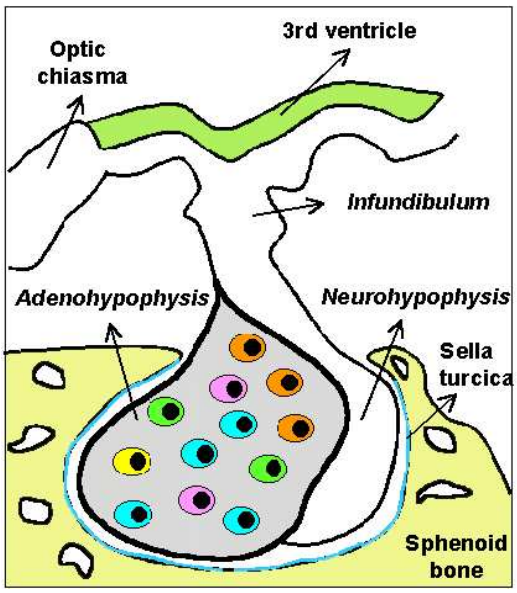

Fig.1. Anatomic location of the hypophysis. The pituitary gland is shown residing in the invagination of the sphenoid bone, the *sella turcica (blue)*, under the *3rd ventricle (green)*. The two lobes, anterior (*adenohypophysis*; in grayish color) and posterior (*neurohypophysis*) are indicated. In the adenohypophysis are shown the 5 hormone-producing cell types: ACTH-producing (orange); GH-producing (blue); PRL-producing (green); FSH/LH-producing (pink); TSH-producing (yellow).

Hormones released from the anterior pituitary gland target and control the function of other systemic endocrine organs. Six main hormones are produced by the adenohypophysis: growth hormone (GH), prolactin (PRL), adrenocorticotrophic hormone (ACTH), follicle stimulating hormone (FSH), luteinizing hormone (LH), and thyroid stimulating hormone (TSH). GH promotes growth of the skeleton and soft tissues and has important metabolic effects. Its effects are mediated directly through GH receptors or indirectly by inducing insulin-like growth factor (IGF-I) synthesis. PRL has important role in the initiation and maintenance of lactation. ACTH stimulates glucocorticoid production from the adrenal cortex. It is split product of proopiomelanocortin (POMC), together β-lipotropic hormone (β-LPH), endorphins, encephalin, corticotropin-like immunoreactive peptide (CLIP), and α-MSH. FSH and LH are collectively referred to as gonadotrophins. FSH promotes follicular growth in the ovaries and spermatogenesis in the testes. TSH is important for the physiological growth and function of the thyroid gland.

It is evident that the adenohypophysis is a complex system of different cell types. Initially the identification of these cell types was based on the reaction of the cells to

staining procedures, which were subsequently divided into basophils, acidophils, and chromophobes. Nowadays with the advances in immunohistochemical and ultrastructural techniques five types of cells are distinguished: GH and/or PRL-producing cells belonging to the acidophilic category; and the basophilic ACTH, TSH and FSH/LH- producing cells. Apart from the hormone producing/endocrine cells, the anterior pituitary also contains the folliculostellate cells, which comprise 3-5% of all adenohypophyseal cells (Allaerts et al., 1990). Their name derives from their stellate shape, due to thin cytoplasmatic projections, which extend between surrounding endocrine cells. Folliculostellate cells are distinguished by their immunoreactivity for the S-100 protein, a low molecular weight soluble protein first isolated from the brain and initially believed to be exclusively a glial marker. Although the actual function of folliculostellate cells still remains unknown, recent extensive studies have shown that they are source of growth factors and cytokines, therefore suggesting an important role in the paracrine regulation of hormone secretion (Schwartz and Cherny 1992; Renner et al., 1996).

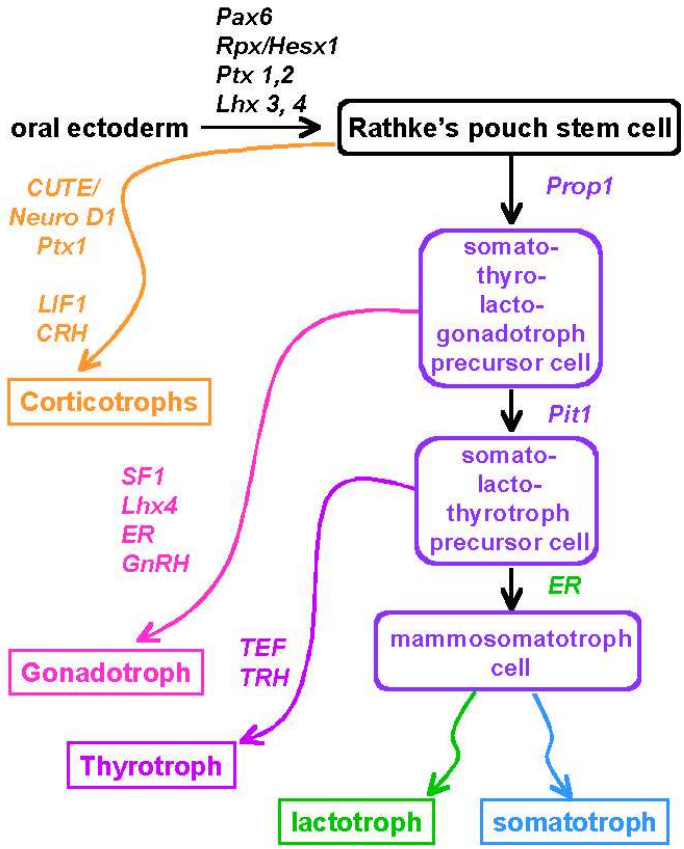

Fig.2. Brief schematic presentation of the major steps in pituitary development and the major transcription factors involved in each. From the oral ectoderm, the Rathke's pouch stem cells arise, one branch of which will give rise to corticotroph lineage and the other to the precursors of the gonadotroph, lacto- somatotroph, and thyrotroph cell lineages. Expression of SF1 in some of these cells will commit them to the gonadotroph lineage, while Pit1 is expressed in the precursor from which the thyrotroph and mammosomatotroph cells will derive. ER: estrogen receptor.

Pituitary adenomas

Pituitary adenomas are composed of adenohypophyseal cells and comprise 15% of all intracranial tumors. The term adenoma refers to a benign glandular tumor, in which the neoplastic cells remain clustered together in a single mass and do not metastasize. Although they are usually benign, they can give rise to severe clinical syndromes due to the hormonal excess they produce, or to visual/ cranial disturbances because of their considerable intracranial mass. The high clinical importance together with the peculiar biological characteristics they display, on one hand, and the obscurity that is covering the differentiation of such a complex cellular system as the adenohypophysis, on the other, made pituitary adenomas the center of intensive study during the last decades. However, as will be extensively described in the following chapters, very few factors are known to be responsible for the pathogenesis of pituitary adenomas, making the search of genes that could be implicated, in the one or the other way, in pituitary tumorigenesis, an issue of high importance.

Classification of pituitary adenomas

A pituitary adenoma can be classified according to the clinical presentation (*functional classification*), tumor size and local invasion (*anatomical classification*), or histology and cytology (*histological examination*).

Functional classification

Pituitary adenomas can be classified into clinically functioning or non-functioning, depending on whether the adenoma development leads to an endocrine syndrome. Clinically functioning pituitary adenomas are the GH-producing adenomas or

somatotrophinomas, prolactinomas, ACTH-producing pituitary adenomas or corticotrophinomas, thyrotrophinomas, and the rare cases of clinically active gonadotrophinomas.

In the case of somatotrophinomas over-secretion of GH and the subsequent increase in IGF-I levels, lead to the acromegaly syndrome, which is characterized by bone expansion in the extremities and progressive disfigurement. Additional symptoms are hypertension, megalocardia, insulin resistance and diabetes mellitus. Treatment with somatostatin analogues improves the clinical status of acromegalic patients by suppressing GH secretion, and in many cases it causes tumor shrinkage, which can facilitate the complete removal of the tumor during surgery without interfering with adjacent structures (Stewart, 2000; Melmed et al., 1998).

Prolactinomas are considered as the major cause of hyperprolactinaemia. Hyperprolactinaemia is causing hypogonadism with subsequent infertility, sexual dysfunction and osteoporosis. Prolactinomas are the most frequent occurring pituitary adenomas and are usually microprolactinomas. The incidence is higher in women, but when occurring in men, these tumors tend to be macroadenomas, with high degree of invasion. Most prolactinomas can be successfully treated after administration of dopamine agonists (Colao et al., 2000). However there are cases with resistance to dopamine treatment, i.e. there is no normalization of serum PRL levels after three months of treatment (Colao et al., 1997). The reason for dopamine resistance was demonstrated to be the low expression or total absence of dopamine D2 receptor in the tumoral cells (Caccavelli et al., 1994).

ACTH-secreting adenomas are associated mainly with Cushing's disease and less frequently with Nelson-Salassa syndrome. Corticotrophinomas present with hyperfunction rather than mass effect, and they are mostly microadenomas. ACTH hypersecretion leads to hypercortisolism which in turn is responsible for the

symptoms of the Cushing's syndrome. Most patients have upper body obesity, rounded face, increased fat around the neck, thinning arms and legs, thin and fragile skin which bruises easily. Fatigue, irritation, anxiety and depression are common psychological findings in these patients (Boscaro et al., 2001). Nelson syndrome-associated corticotrophinomas are larger and more aggressive. They result from the lack of glucocorticoid feedback due to prior adrenalectomy (Sonino et al., 1996). In the absence of efficient medical therapy, transsphenoidal adenomectomy is still the treatment of choice for corticotrophinomas.

TSH-secreting adenomas or thyrotrophinomas are very rare and usually present with mass effect symptoms and sometimes with signs of hyperthyroidism. Transsphenoidal surgery is the treatment of choice although octreotide treatment has been shown to normalize TSH levels and to cause tumor shrinkage (Beck-Peccoz et al., 1996).

The non-functioning pituitary adenomas, which comprise 25% of all pituitary adenomas, do not lead to any endocrine syndrome and they present with symptoms of an intracranial mass, such as headache and visual field defects. Transsphenoidal surgery is the first approach in these adenomas, in order to remove the tumor mass and post-operative radiotherapy is applied to prevent tumor regrowth (Snyder, 1995). However, radiotherapy has as big side effect the occurrence of hypopituitarism, therefore development of proper medical therapy would be useful as a less risky alternative to radiotherapy (Colao et al., 1998).

Anatomical classification

Neuroradiological examination provides information about the tumor size and extent of local invasion. Adenomas are classified in four grades (Hardy's classification; Hardy J, 1979): *Grade I* refers to microadenomas, i.e. <10mm in diameter; *Grade II*

refers to macroadenomas (>10 mm in diameter) that may exhibit suprasellar extension but no invasion to the surrounding bony structures; *Grade III* adenomas are locally invasive tumors; and *Grade IV* refers to large invasive tumors that can involve, except the bone, the hypothalamus and the cavernous sinuses.

Histological classification

The advances in immunohistochemistry and electron microscopy led to the development of a classification system based on the hormonal content of each pituitary adenoma and the special cytological structures it may exhibit. Using specific antibodies against each hormone it became evident that acromegaly-associated pituitary adenomas can also produce PRL (*mammosomatotrophinomas* and *mixed GH-PRL adenomas*) and that some prolactinomas display GH reactivity (‘*acidophilic stem cell adenomas*). However the biggest insight was given in the class of the non-functioning pituitary adenomas. Although most clinically inactive adenomas are *gonadotrophinomas*, i.e. show reactivity for either FSH, LH or α-subunit, or a combination of the three, there are cases that are negative for all the hormones and for α-subunit (*null cell adenomas*). However it is of interest that many null cell adenoma cases were found to express mRNA for the alpha subunit, FSH and LH (Lloyd et al., 1991). An intriguing class presents adenomas that, although clinically inactive show reactivity for ACTH and are therefore referred to as *silent corticotrophinomas*.

Ultrastructural examination by electron microscopy had subdivided each category according to special characteristics in the cell morphology of each case. By this way, GH and PRL producing tumors are further classified into densely and sparsely granulated somatotrophinomas, according to the density of secretory granules, and null cell adenomas are divided into *oncocytomas*, which are characterised by an

intracellular accumulation of large numbers of dilated mitochondria, and pure *null adenomas*.

In the following chapters, the pituitary adenomas will be classified mainly according to their clinical phenotype and, when necessary, according to their histological findings. The classification follows a clinicopathological scheme which permits a more complete characterization and understanding of each case since combining the clinical and the histological data can facilitate the interpretation of the results derived from the research of the etiology of pituitary adenomas.

Receptors in pituitary adenomas

The pituitary gland is under continuous control of peptides and proteins originating from the hypothalamus and the periphery. The stimulatory or inhibitory effect of these substance is mediated upon binding to cell surface or nuclear receptors. Since the same receptors mediate the effects of synthetic pharmaceutical compounds, an understanding of their biological and biochemical properties is important for the proper design and application of different drugs. Two receptors have drawn attention in the study of pituitary physiology, the dopamine type 2 receptor (D2R) and the somatostatin receptors (SSTR), since they mediate the effects of the two most commonly used, for the treatment of pituitary adenomas, families of drugs, the dopamine agonists and the somatostatin analogues. In this thesis we will concentrate to one aspect of the action of somatostatin analogues.

SSTR. Somatostatin is mediating its effect upon binding to a family of five receptors (SSTR1-5) which belong to the seven transmembrane G-protein coupled receptor superfamily. SSTR2 is present as two isoforms: SSTR2A and B, which are generated by alternate mRNA splicing (Patel et al., 1993). All SSTRs are coupled to Gi protein and inhibit adenylate cyclase and cAMP accumulation. In addition, somatostatin

signaling involves tyrosine phosphatase activation and ion channel conductance (Patel et al., 1995).

SSTR2 and 5 are the main receptors found in the adenohypophysis (Shimon et al., 1997a). SSTR2 is the predominant form in the somatotrophs, but is also present in many corticotrophs and gonadotrophs (Shimon et al., 1997a; Mezey et al., 1998). SSTR5 is the predominant SSTR in lactotrophs. In pituitary adenomas, SSTR2 mRNA was found in the majority of acromegaly-associated tumors and in a significant number of non-functioning adenomas, while there was no expression in prolactinomas and corticotrophinomas (Greenman and Melmed, 1994a). On the other hand, SSTR5 is present in most GH-secreting tumors and in all prolactinomas. High levels of SSTR3 mRNA were detected in most tumors regardless class, while SSTR4 was undetectable (Greenman and Melmed, 1994b).

Native somatostatin binds to all the five receptor forms with high affinity. Search for more potent analogues resulted in the development of octreotide (SMS-201-995) and later of lanreotide (BIM-23014) and octastatin (RC-160) (Hofland et al., 1995). All these three analogues have high affinity for SSTR2 and a moderate one for SSTR3 and 5. Analogues specific for the other SSTR subtypes have been recently developed (Shimon et al., 1997b).

Somatostatin, far from inhibiting GH secretion, has a wider spectrum of inhibitory action on the adenohypophysis and other neuroendocrine tumors (Lamberts et al., 1991). Octreotide and lanreotide are the most frequently used analogues in the management of acromegaly and of a number of neuroendocrine tumors, such as carcinoid tumors and endocrine pancreatic tumors (Lamberts et al., 1996; Eriksson and Oberg, 1999). The therapeutical value of somatostatin analogues in these types of cancer is due to the inhibitory action on hormone production from the tumors. However, there is increasing evidence from in vitro studies of an antiproliferative role

for octreotide in several tumors, which highlight the potential use of somatostatin analogues as anti-tumor agents, and pave the way for the search and development of more potent analogues in cancer therapy.

Molecular basis of pituitary tumorigenesis

General mechanisms of cancer development

Genetic instability is necessary for the genesis and progression of cancer. Cell transformation and abnormal proliferation results from a disruption in one or more regulators of cell cycle progression and apoptosis. This can be an activation of a promoter of cell growth (*proto-oncogene*) or an inactivation of a blocker of cell growth/ inducer of apoptosis (*tumor suppressor gene; TSG*).

Proto-oncogenes are the cellular counterparts of the oncogenes, which when overexpressed cause tumor formation. Proto-oncogene products are involved in mediating signals from the cell surface to the nucleus, like protein tyrosine (e.g. src), serine/threonine kinases (e.g.raf) and G proteins (e.g. ras, gsp), cell surface receptors (e.g. c-erbB2/*neu*), transcription factors (c-myc, c-fos, c-myb), and cell cycle regulators (e.g. cyclin D, mdm2). Proto-oncogenes can be transformed to oncogenes and lead to tumor formation by an activating mutation or gene overexpression.

The other mechanism that can lead to cancer development is functional inactivation of a TSG. According to the classical model of recessive oncogenesis, a genetic mutation in the coding region of the gene is required to inactivate the TSG but is not sufficient (therefore the term *recessive*). An additional alteration in the remaining allele, a 2nd hit, is necessary for the TSG inactivation to occur (Knudson's *two-hit* hypothesis; Knudson, Jr., 1975). In the classical Knudson's model the 2nd hit is a loss of heterozygosity (LOH), i.e. loss of the wild-type, non-mutated allele. However, it

was soon found out that TSG mutations in sporadic cancers are rare and the seek of alternative mechanisms of gene silencing began.

DNA methylation

An alternative to coding region mutation was found to be hypermethylation. Methylation is an epigenetic modification of the genome, in which a methyl group is added to the 5-position of cytosine within the context of the dinucleotide palindrome CG (commonly referred to as CpG, with p denoting the phosphate group) (Bird, 1992). CpG are not randomly distributed (Cooper and Krawczak, 1989), but rather clustered in the 5' ends of genes in the so called 'CpG islands'. Approximately 60% of genes have CpG islands in their promoters which are normally unmethylated. In normal mammalian cells, DNA methylation is responsible for the silencing of the inactive X chromosome (Lee and Jaenisch, 1997) and of genes subject to parental imprinting (Barlow, 1995).

Hypermethylation was demonstrated either as an alternative to LOH for the 2nd hit in the Knudson's model of tumorigenesis or as the sole reason of TSG inactivation (Fig.3). Approximately half of the TSGs responsible for familiar cancer syndromes (e.g. *p16*, BRCA1, E-cadherin) have been reported to be hypermethylated in the sporadic cancers (Herman, 1999). Therefore hypermethylation is now considered to play a major role in inactivating these genes.

Another way that altered methylation can play a role in cell transformation, is unmethylation of the CpG in the promoter of an oncogene. Hypermethylation in many proto-oncogenes is a physiological lock which forbids the activation of these factors and therefore keep the cell proliferation under control (Jones, 1996). Waving the heavy methylation from the promoter of these genes lead to the uncontrolled oncogene expression, and subsequent cell transformation (Fig.3).

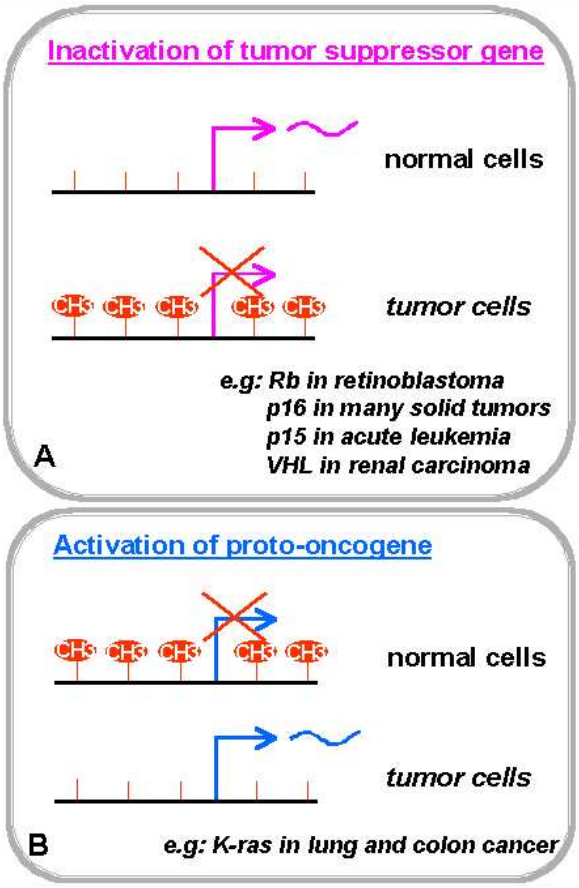

Fig.3. Diagram of the two ways by which hypermethylation can contribute to tumorigenesis. *A*. Hypermethylation of a TSG blocks its expression, resulting in uncontrolled cell growth. *B*. In normal adult cells protooncogenes are not expressed. One mechanism of silencing is heavy hypermethylation of the gene promoter. When this mechanism is altered, there is abnormal expression of the protooncogene resulting in increased cell growth and tumor formation.

The most common TSGs found to play a role in human cancer are parts of the cell cycle machinery and as such, they can control cell proliferation. For example, the retinoblastoma susceptibility gene encodes a nuclear protein (Rb) which binds to nuclear proteins and transcription factors (E2F), preventing them from promoting the expression of genes essential in cell cycle progression. Other TSGs, like p53, p16,

and p27 act by inhibiting the assembly of CDK-Cyclin complexes (Fig.4; Pestell et al., 1999).

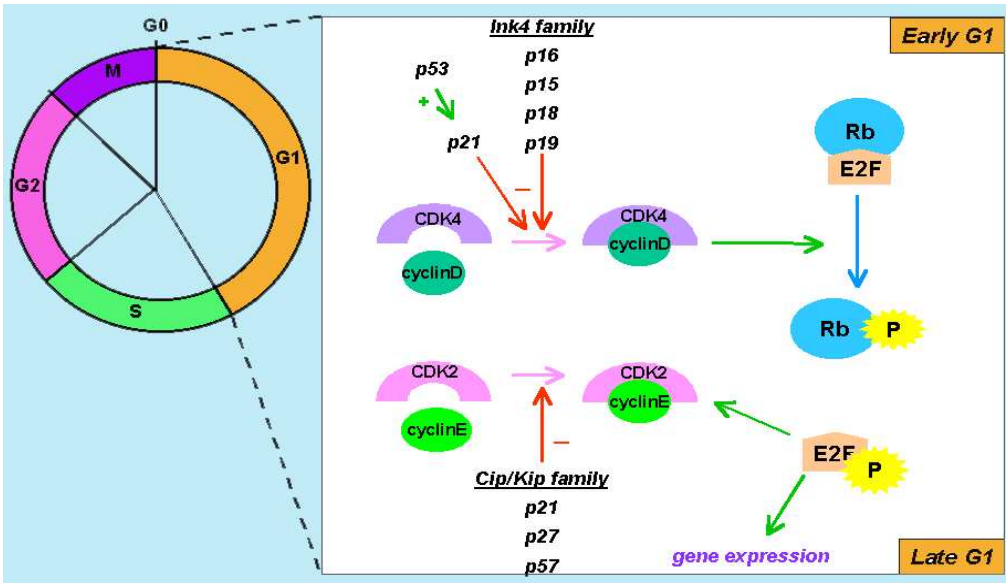

Fig.4. Schematic presentation of the major events taking place in the G1 phase. Many tumor suppressor genes whose expression is lost in cancer (e.g. p16, p27) are exerting their antiproliferative role by inhibiting the assembly of cyclins (e.g. cyclin D and E) to their cyclin kinase counterparts (e.g. CDK4 and 2), therefore blocking the release of factors (e.g. E2F) which in turn would promote the expression of genes involved in cell cycle progression to S phase.

Origin of pituitary adenomas

There were two hypotheses about the origin of pituitary adenomas: the hypothalamic hypothesis versus the monoclonal expansion model (Fig.5). Nowadays, it is widely accepted that pituitary adenomas are monoclonal in origin, i.e. they derive from a single transformed cell (Alexander et al., 1990). However, the issue of monoclonality was recently brought under discussion after a study which revealed that in many cases of recurrence, the second tumor was not having the same LOH status as the original tumor, implying that the second tumor arises from a different clone than the

first one (Clayton et al., 2000). Even though, it has to be kept in mind that in this study each individual tumor specimen was still demonstrated to be monoclonal.

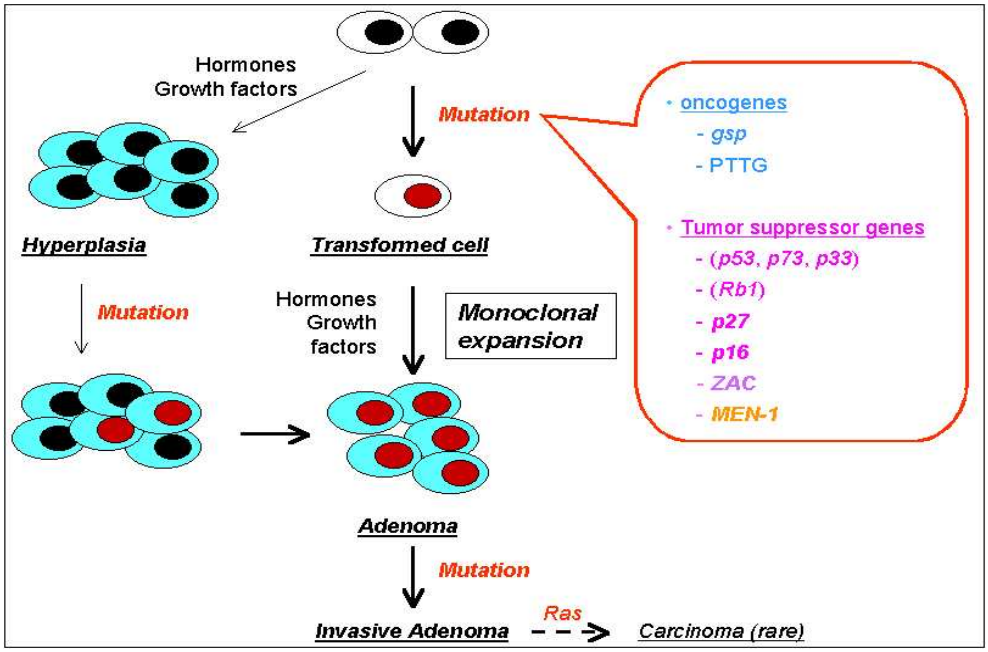

Fig.5. Model of pituitary tumorigenesis. Hypothalamic hypothesis (*left*) *vs.* the monoclonal expansion model (*center*). According to the hypothalamic hypothesis, hormones and growth factors are causing hyperplasia, and on these hyperplastic cells spontaneous mutations lead to adenoma formation. The monoclonal expansion model states that one mutation in one single cell transforms it, and growth factors and hormones acting on this transformed cell enhance its proliferation and the whole procedure results in a pituitary adenoma. In extremely rare cases an additional mutation leads to pituitary carcinoma. In the box shown in the right part of the cartoon, are shown the oncogenes and tumor suppressor genes that have been studied as target genes for the initial 'transforming' mutation.

The monoclonality of pituitary adenomas is still the widely accepted model, according to which genetic events in one cell are enough to transform it and to trigger the adenoma formation (Fig.5). This event can be an activating mutation in a proto-oncogene or an inhibiting mutation in a TSG. Somatic mutations frequently found in other malignancies, such as mutations involving *ras*, c-erbB2/*neu*, and p53, are absent in the majority of pituitary neoplasias.

LOH studies in pituitary adenomas have revealed that the long arm of the chromosome 11 (11q13) at the *MEN1* locus is lost in a significant number of cases (Boggild et al., 1994; Prezant et al., 1998; Tanaka et al., 1998; Farrell et al., 1999). Loss of the *RB1* gene locus in the long arm of chromosome 13 (13q) was detected in invasive tumors and pituitary carcinomas (Pei et al., 1995; Simpson et al., 1999a), while LOH affecting the long arm of chromosome 10 (10q26) is also detected in invasive tumors (Bates et al., 1997). On the other hand, LOH on the short arm of chromosome 9 is present in about 30% of pituitary tumors, independent of their invasiveness (Farrell et al., 1997). In general, incidence of LOH in 11q13, 13q and 10q26 was found to be higher in invasive than non-invasive adenomas suggesting that inactivation of these TSGs is responsible for tumor progression but not tumor initiation (Bates et al., 1997).

As it will be evident from the following chapters, there is a large number of studies dedicated to reveal genetic alterations and defects in the expression of oncogenes and TSGs known to be involved in the genesis and progression of other types of cancer. However, despite the extensive invetsigation the pathogenetic mechanisms driving pituitary tumorigenesis still remain obscure.

Protooncogenes

G proteins. G proteins play an important role in transducing signals from cell surface ligand-receptor complexes to downstream effectors (Simon et al., 1991). Three types are the best known in cell signaling: the stimulatory Gs, which is involved in the GHRH and CRH pathways, the Gq, in the TRH and GnRH signaling, and the inhibitory Gi, in the somatostatin and dopamine pathways. They are composed of three subunits, α, β, and γ, which dissociate when GTP binds. The dissociated α subunit of Gs (Gsα) stimulates adenylate cyclase, leading to cAMP production,

which in turn, activates cAMP-depended protein kinases. GTPase activating peptides release GTP from Gsα, terminating the whole cascade. Activating mutations in the Gsα inhibit the GTP hydrolysis and maintain Gsα in a constitutively activated state. Approximately 40% of somatotrophinomas contain mutations in the Gsα proteins, or *gsp*, that substitute at arginine 201 or glutamine 227 (Landis et al., 1989). Recently it was observed that although Gsα is monoallelically expressed in the normal human pituitary, it is biallelically expressed in the tumors, and that activating mutations occur on the more highly expressed maternal allele (Hayward et al., 2001).

Cyclin D/ CCND1. Cyclin D1 plays an important role in the regulation of cell progression through the G1 phase of the cell cycle. The cyclin D1 gene (*CCND1*) is located on chromosome 11q13, which is often found to be rearranged in pituitary tumors. Cyclin D was not found in normal pituitary, but it was observed in 67% of the non-functioning pituitary adenomas and 37% of somatotrophinomas, suggesting that its overexpression can lead to tumor formation (Hibberts et al., 1999).

PTTG. Pituitary tumor transforming gene (*PTTG*) was isolated from the rat pituitary tumor GH4 cell line by differential RNA display (Pei and Melmed, 1997). *PTTG* was found to be highly expressed in several cancers, implying that it is a common and important factor in malignancy (Heaney et al., 2000; Saez et al., 1999). RT-PCR revealed low levels of PPTG expression in human normal pituitary and high levels of expression in the pituitary adenomas, especially in Grade III and IV hormone secreting pituitary tumors (Zhang et al., 1999a). One possible mechanism of PTTG action is proposed to be through activation of bFGF, which is a potent mitogenic and angiogenic factor (Zhang et al., 1999b; Heaney et al., 1999), or through activation of the c-myc oncogene (Pei, 2001). In additon, PTTG is identical to human securin,

which is involved in sister chromatid separation (Zou et al., 1999; Wang et al., 2001). Therefore it is possible that the oncogenic potential of PTTG derives from its ability to block chromatid separation, resulting in increased chromosome instability.

Tumor suppressor genes

Rb1. Rb1 was suspected as a candidate TSG in pituitary tumor formation, when mice heterozygous for a *RB1* mutation were shown to have a complete predisposition to pituitary tumors derived from the intermediate lobe (Jacks et al., 1992; Hu et al., 1994). Although LOH studies revealed loss of the *RB1* locus only in invasive pituitary adenomas and in the rare cases of pituitary carcinomas, in the vast majority of the pituitary adenomas the RB1 locus seems to be intact (Zhu et al., 1994; Pei et al., 1995; Bates et al., 1997). However, in a recent study, 27% of somatotrophinomas and 4% of the non-functioning tumors were not expressing pRb (Simpson et al., 1999a). No mutation in *RB1* gene was detected, and the reason for the loss of Rb1 in these tumors was found to be hypermethylation of the *RB1* promoter (Simpson et al., 2000). These last findings bring again into discussion a role of Rb1 in the pathogenesis of, at least, a fraction of pituitary adenomas, and modify the classical Knudson's two-hit hypothesis for the few cases with LOH in the RB1 locus, by substituting gene mutation with promoter hypermethylation.

p53. *p53* encodes a nuclear protein, which regulates the CDK inhibitor p21 (Fig. 3), and induces cell cycle arrest at the G1/S checkpoint and apoptosis. Although it is the most frequently mutated gene in human cancers (Hollstein et al., 1991), the studies performed failed to detect mutation of this gene in pituitary tumors. Mice deficient for both *RB1* and *p53* develop pituitary tumors (Harvey et al., 1995). p53 protein expression was found in a small fraction of invasive adenomas and in carcinomas

(Thapar et al., 1996), while in another report, abnormal p53 protein accumulation was described in a high percentage of Cushing's and invasive non-functioning adenomas (Buckley et al., 1995). In a later study, the nuclear p53 accumulation in the corticotrophinomas was associated with a significantly lower apoptotic index (Kontogeorgos et al., 1999).

p16. This protein belongs to the INK4 (Inhibitor of CDK4) group of CDK inhibitors, which mainly inactivate the cyclinD/CDK4 complex, preventing it from phosphorylating pRb and progress to S phase. *p16* is inactivated in many cancers, either by homozygous deletion, or because of hypermethylation induced gene silencing. p16 was found to be lost in the majority of pituitary adenomas, a finding that was not associated with gene mutation or gene loss (Woloschak et al., 1996). The mechanism lying behind *p16* loss was found to be hypermethylation of the gene promoter (Farrell et al., 1997). A subsequent study has pointed the significant prevalence of hypermethylated *p16* in non-functioning pituitary adenomas (70%) versus somatotrophinomas (9.5%; Simpson et al., 1999b). The latest showed no correlation between the degree of hypermethylation and tumor invasiveness, indicating that hypermethylation and subsequent decrease in p16 expression is an early event in the oncogenesis of this type of pituitary tumors.

Recently using the mouse corticotroph cell line AtT-20 the antiproliferative role of p16 has been confirmed in pituitary cells. In this cell model, p16 is lost due to homozygous deletion. Transfection of AtT-20 cells with an inducible p16 construct resulted in G1 arrest and reduced proliferation (Frost et al., 1999).

p27. Attention was drawn to this gene after the observation that mice with homozygous deletion of p27 develop pituitary tumors of the intermediate lobe (Fero

et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). In addition, mice haplo-insufficient for p27, show hyperplasia of the intermediate lobe and are sensitive to irradiation and carcinogens (Fero et al., 1998). Double knockout mice for p27 and p18 developed pituitary tumors in the intermediate lobe much earlier when compared to the p27 knockout mice (Franklin et al., 1998). This finding, together with the high levels of p27 and p18 expression found in the pituitary gland, point to a collaboration between the two CDK inhibitors in suppressing pituitary tumorigenesis. On the other hand, null mutation for cyclin D1 in the p27 null mice, had no effect in averting the development of pituitary tumors, suggesting that p27 acts independent of cyclin D1in pituitary tissues (Tong and Pollard, 2001).

These studies triggered a number of investigations to examine the status of p27 in pituitary adenomas. However, in pituitary tumors no p27 gene mutations were found and the levels of *p27* gene transcript were shown to be comparable to that of the normal pituitary gland (Ikeda et al., 1997; Jin et al., 1997; Dahia et al., 1998; Takeuchi et al., 1998). However, when normal and adenomatous pituitary were analyzed by immunohistochemistry, p27 protein levels were lower in pituitary tumors than in normal pituitary (Jin et al., 1997; Lloyd et al., 1997; Bamberger et al., 1999). The reduction of p27 protein was predominant in corticotrophinomas and in metastatic tumors (Lidhar et al., 1999). The significant reduction of p27 protein in corticotrophinomas imply a significant role of p27 in the development of this type of pituitary adenoma.

These observations fail to place p27 in the classical two-hit model for TSG. However, they point out a new mechanism of TSG regulation, in which alterations postranslational level are enough to de-activate the TSG product. In other types of tumors, it is now proven that abnormalities in the ubiquitin-mediated degradation

system are responsible for the down-regulation of p27 protein (Pagano et al., 1995; Loda et al., 1997).

MEN1. *MEN1* is the gene mutated in families with multiple endocrine syndrome 1 (MEN1). This autosomal dominant disorder is characterized by neuroendocrine tumors of the pituitary, parathyroid, pancreas, and duodenum, and less frequently by tumors of the adrenal and thyroid gland, angiofibromas, leiomas, and lipomas (Marx et al., 1999; Pannett and Thakker, 1999). The susceptibility locus was mapped to chromosome 11q13 (Larsson et al., 1988), and ten years later, the gene was identified by positional cloning (Chandrasekharappa et al., 1997; Lemmens et al., 1997). *MEN1* encodes a 610-amino acid protein termed 'menin', which has neither homology to any known protein nor any conserved motifs. Menin is widely expressed in most adult tissues (Chandrasekharappa et al., 1997) and is predominantly located in the nucleus (Guru et al., 1998). Menin functionally interacts with the AP1 transcription factor junD (Agarwal et al., 1999) and suppresses the *RAS*–mediated tumor phenotype (Kim et al., 1999), indicating that it may play a role in the cell cycle regulation. An association between menin expression and cell-cycle has been suggested (Kaji et al., 1999), and an inhibitory effect of menin on DNA synthesis has been demonstrated under DNA damaging conditions (Ikeo et al., 2000). Recently menin was shown to interact with the TGF-β effector Smad3, preventing complex formation with Smad4 and subsequently inhibiting its transcriptional activity (Kaji et al., 2001). Menin was also shown to interact with the putative tumor metastasis suppressor nm23 (Ohkura et al., 2001) and with the NF-kappaB transcription factor (Heppner et al., 2001) These studies, together with the fact that *MEN1* mutations are of the loss-of-function type and that the wild type allele is lost in tumors derived from MEN1 affected patients, make *MEN1* a candidate tumor suppressor gene.

Although *MEN1* mutations are found in around 30% of sporadic pancreatic tumours (Zhuang et al., 1997a; Toliat et al., 1997), and 20% of sporadic parathyroid tumours (Heppner et al., 1997), they appear extremely rarely, if at all, in sporadic pituitary adenomas (Zhuang et al., 1997b; Prezant et al., 1998; Schmidt et al., 1999; Wenbin et al., 1999). LOH in the 11q13 locus has been described in 5-20% of sporadic pituitary adenomas (Boggild et al., 1994), and gene expression is not down-regulated in the pituitary tumors (Asa et al., 1998; Prezant et al., 1998; Farrell et al., 1999). However, these studies do not address the issue of possible alterations at the protein level. To address this issue menin expression was examined in a series of tumor cell lines and tissues using Western blot. However, there is no *in situ* analysis of menin expression in normal and tumoral tissue due to the lack of an antibody suitable for immunohistochemistry. The importance of an *in situ* analysis is evident if we take into account that techniques like western blot don't exclude the probability of contamination with normal tissue and cannot differentiate between endocrine and non-endocrine parts of the tumor, such as fibroblasts and vessels. In addition, it is important to be able to assess which cell types in the organ express menin in physiological and pathological stages. Recently an antibody was developed, which was proven to be efficient for immunohistochemistry and immunofluoresence (Ferro, et al 2001). Using this antibody, a series of normal and adenomatous pituitary was studied by immunohistochemistry, as will be described in the RESULTS section.

ZAC. *ZAC* (zinc finger protein inducing apoptosis and cell cycle arrest) is the human homologue of *Zac1* which was isolated from the AtT20 mouse corticotrophinoma cell line by a functional expression cloning technique, upon its ability to induce the type I pituitary adenylate cyclase activator protein (PACAP) receptor, together with *p53* (Spengler et al., 1997). It was found that ZAC/Zac1 has antiproliferative properties

related to its ability to induce apoptosis and cell cycle arrest, establishing this candidate TSG as the first gene structurally unrelated to *p53* that shares its apoptotic and cell cycle blocking functions. The same gene was independently cloned in a rat model of epithelial ovarian cancer and was named *Lot1* (*l*ost *o*n *t*ransformation) because it was lost in transformed rat ovarian surface epithelial cell lines (Abdollahi et al., 1997).

ZAC/*LOT1* is mapped to the chromosome 6q24-25 (Abdollahi et al., 1997) (Varrault et al., 1998), a region frequently lost in ovarian, breast, kidney and pleural mesothelial cancers (Fujii et al., 1996; Taguchi et al., 1993; Theile et al., 1996; Thrash-Bingham et al., 1995), and its expression was found to be reduced in breast (Bilanges et al., 1999) and ovarian (Abdollahi et al., 1997) cancer cell lines and primary tumors. Searching for a mechanism responsible for loss of the gene in ovarian cancer, it was shown that in normal rat ovarian surface epithelial cells the EGF receptor ligands, EGF and TGFα, were able to down-regulate *Lot1* expression, and this effect was reversed after blocking the EGF receptor signaling pathway (Abdollahi et al., 1999).

Another mechanism that can be responsible for the reduced levels of *ZAC* is the aberrant gene hypermethylation. It was observed that the treatment of breast cancer cell lines with the methylation interfering agent 5-azacytidine enhanced *ZAC* gene expression (Bilanges et al., 1999). A number of studies have demonstrated that *ZAC* is an imprinted gene (Piras et al., 2000; El Kharroubi et al., 2001), i.e. the copy of the gene inherited by the mother is silenced by hypermethylation and only the paternal copy is expressed. In addition, *ZAC* was found to reside in an imprinted locus associated with transient neonatal diabetes mellitus and suggested as a candidate gene for this disease (Kamiya et al., 2000; Arima et al., 2000). The pitfall with this studies was that the coding region of the gene was too far from the imprinted locus.

Finally, the complete characterization of the gene revealed that it is composed of nine exons, from which only the exon VIII and IX are translated, and that part of exon I and the promoter are located in a CpG rich region of the chromosome 6q24 (Varrault et al., 2001). *ZAC* expression is monoallelic, therefore a single 'hit', the inactivation of the paternally expressed allele, would be sufficient to down-regulate the gene. Taking into account all these data, it is possible that hypermethylation is one of the mechanisms responsible for the reduced levels of ZAC in the different types of cancer.

ZAC/Zac1 has a wide tissue distribution (pituitary, kidney, placenta, adrenals), but it is most highly expressed in the pituitary gland (Varrault et al., 1998). *Zac1*-expression, as studied by *in situ* hybridization, was found to be high in the anterior lobe of the mouse pituitary, moderate in the intermediate and very weak in the posterior lobe (Pagotto et al., 1999). Ablation of the endogenous Zac1 protein by antisense targeting in murine tumoral pituitary cell lines resulted in enhanced DNA synthesis, further confirming the antiproliferative role of *Zac1* and demonstrating a role for *Zac1* in pituitary cell proliferation (Pagotto et al., 1999).

The antiproliferative properties of ZAC together with its high expression in the pituitary gland led to the speculation that ZAC may play a role in pituitary tumorigenesis. Studies on ZAC gene status in pituitary adenomas revealed LOH in 8 out of 18 pituitary adenomas, but no mutations in the ZAC coding region (Pagotto et al., 2000). However, there is still a possibility of a defect in the transcriptional regulation of the gene. To address this issue, the expression of ZAC at mRNA and protein levels was studied in a series of pituitary adenomas as will be described in the RESULTS section.

Other factors involved

COUP-TFI. Chicken ovalbumin upstream promoter-transcription factor or COUP-TF is an orphan receptor that belongs to the steroid/thyroid hormone receptor superfamily. It was first identified as a homodimer that binds to a direct repeat element in the chicken ovalbumin promoter (Sagami et al., 1986). Two COUP-TF genes were cloned from human cells, COUP-TFI (Wang et al., 1989) or EAR3 (Miyajima et al., 1988) and COUP-TFII (Ritchie et al., 1990; Wang et al., 1991) or ARP-1 (Ladias and Karathanasis, 1991). COUP-TF homologues have been cloned from many species, including drosophila and mouse, and there is a high degree of homology between species, from metazoans to human, implying that these genes play an important, and maybe vital, role in cellular function (Qiu et al., 1994; Tsai and Tsai, 1997). Indeed, in Drosophila, mutation of the COUP-TF homologue (seven-up gene or *svp*) is lethal. In zebrafish, chicken, and mouse, COUP-TFs are expressed at high levels in the developing central nervous system, while in mouse they are highly expressed in many developing organs (Jonk et al., 1994); (Pereira et al., 1995), suggesting an important role during embryonic development. Null mutation of COUP-TFI resulted in defects in neurogenesis and axon guidance (Qiu et al., 1997), while targeted disruption of COUP-TFII resulted in embryonic lethality due to defects in angiogenesis and heart development (Pereira et al., 2000). In the mouse, COUP-TFI is expressed mainly in the central nervous system and nasal septum, while COUP-TFII shows a wider organ distribution, including central nervous system, testis, ovary, skin, lung, stomach, intestine, pancreas and salivary gland (Qiu et al., 1994; Pereira et al., 1995). In addition, COUP-TFII immunoreactivity is observed mainly in mesenchymal cells, but is absent in terminally differentiated epithelium (Suzuki et al., 2000), supporting the hypothesis that it plays an important role in the induction of

epithelial differentiation (Tsai and Tsai, 1997). In adult mice, the expression of COUP-TFs is dramatically reduced and restricted mainly to distinct parts of the brain, which are the olfactory nucleus, neocortex and parts of hippocampus and cerebellum for COUP-TFI, and the reticular and thalamic nuclei for COUP-TFII (Tsai and Tsai, 1997).

All these data point to a role of COUP-TFs in development and differentiation. However none of the studies had related COUP to pituitary function and pathology. Attention was drawn on COUP-TFI during an investigation demonstrating the ability of retinoic acid to prevent experimental Cushing's syndrome. Retinoic acid was shown to inhibit POMC promoter and consequently ACTH biosynthesis in the mouse corticotrophinoma cell line AtT-20 (Paez-Pereda et al., 2001). In addition, retinoic acid caused a significant inhibition of ACTH production in the majority of corticotrophinomas studied in primary cell culture but had no effect on ACTH produced by rat normal pituitary cells, indicating a tumor-specific effect. Searching for transcription factors that could affect retinoic acid-inducible transcription, COUP-TFI was the factor that readily came in mind, since it is a well known transcriptional repressor of vitamin D, thyroid and retinoic acid receptor signaling (Kliewer et al., 1992; Tran et al., 1992; Cooney et al., 1993), and POMC promoter has two COUP elements, one within the glucocorticoid binding site and the other adjacent to the AP-1 binding site (Therrien and Drouin, 1991).

As will be shown in the *Results* section, RT-PCR and immunohistochemistry revealed COUP-TFI expression in the human normal pituitary gland but not in Cushing-associated pituitary adenomas, therefore providing, on one hand, a basis for the differential response of normal and adenomatous corticotrophs to retinoic acid and, on the other hand, introducing a candidate that may play an important role in the differentiation of corticotrophs and the pathogenesis of corticotrophinomas.

Growth factors

Growth factors are soluble peptides with critical functions in mitogenesis, angiogenesis, and gene transcription. The pituitary gland has been shown to be a reservoir of growth factors, which act in an autocrine/paracrine fashion to regulate pituitary cell proliferation and hormone secretion (Renner et al., 1996; Ray and Melmed, 1997). The uncontrolled mitogenic function of growth factors is one of the mechanisms leading to tumorigenesis. Possible alterations in the expression of growth factors and/or their receptors in pituitary tumors are subject of ongoing research, aiming to unravel their possible role in pituitary oncogenesis. Later in this thesis, we will concentrate on the epidermal growth factor (EGF) and its receptor (EGFr).

EGF acts as a mitogen in a broad variety of endothelial and epithelial cell types. In the pituitary, EGF is detected in most adenohypophyseal cells (LeRiche et al., 1996) and is a potent stimulator of PRL (White and Bancroft, 1983) and ACTH (Childs et al., 1995) release. EGF exerts its effects after binding to a tyrosine kinase receptor, which is the cellular homologue of the *v-erbB* oncogene product (Downward et al., 1984). EGF receptor (EGFr) is overexpressed in a wide range of tumors, such as breast and ovarian cancer and glial cell tumors (Klijn et al., 1992) (Xu et al., 1984). Despite the fact that there is clear evidence about the presence of EGFr in the normal adenohypophysis (Chabot et al., 1986; Fan and Childs, 1995), the data concerning the expression of EGFr in the pituitary tumors are controversial (reflected in the two recent reviews of Ray and Melmed (1997) and Dahia and Grossman (1999). Initial studies had failed to show any EGF binding sites in human pituitary adenomas (Birman et al., 1987), while further investigation using immuno-histochemical and RT-PCR approach described EGFr in pituitary adenomas, with

one demonstrating overexpression in most non-functional pituitary adenomas but absence in the hormone-secreting types (Chaidarun et al., 1994), and the other showing EGFr expression in all types of pituitary adenomas, but at varying levels (LeRiche et al., 1996). This last paper had also related EGFr-expression with tumor aggressiveness. A recent report has revealed high levels of EGF-binding sites in macroprolactinomas, also suggesting a link with tumor invasiveness (Jaffrain-Rea et al., 1998).

AIM OF THE STUDY

The group of studies described herein aim to understand the role of transcription factors, which are known or suspected to play an important role in cancer, in the pathogenesis of certain types of pituitary adenomas.

Three factors were selected for the current study: ZAC, menin, and COUP-TFI. ZAC was chosen because of its high levels of expression in the pituitary gland and its proven antiproliferative effects in a mouse model of pituitary tumor. Menin was selected because one of the major manifestations of familiar MEN1 syndrome is pituitary adenoma formation. Therefore it is an open question whether alterations in *MEN1* expression can play a role in the formation of sporadic pituitary adenomas. Since *MEN1* gene and its expression are intact in sporadic pituitary adenomas, the present work seeks to examine possible alterations in the status of the *MEN1*-gene product, menin. The implication of COUP-TFI in pituitary pathogenesis was suspected during a study searching for a mediator of the retinoic acid's anti-tumorigenic effects in tumoral but not in normal corticotroph cells.

All the above mentioned genes were studied at the mRNA level using reverse transcription-PCR and *in situ* hybridization and, at protein level, using immuno-histochemistry. In the case of ZAC, mouse cell models of pituitary tumors and human pituitary adenomas in primary cell culture were used in an attempt to elucidate the mechanisms responsible for the regulation of this gene's expression, and to find a way to pharmacologically induce its expression.

MATERIALS & METHODS

Reagents

Product	Company
[α-^{33}P]dATP	NEN (Cologne, Germany)
ABC blocking kit	Vector Laboratories (Burlingane, CA, USA)
Acetic acid	MERCK (Darmstadt, Germany)
Acridine orange	Sigma (St. Louis. MO, USA)
AG 1478	Calbiochem (Darmstadt, Germany)
Agar	Life Technologies (Paisley, Scotland, UK)
Ammonium persulfate	Sigma (St. Louis. MO, USA)
Amphotericin B	Biochrom (Berlin, Germany)
Ampuwa water (for ISH)	Frisenius (Germany)
5-Aza-2´-deoxycytidine	Sigma (St. Louis. MO, USA)
Beta-mercaptoethanol	MERCK (Darmstadt, Germany)
Boric acid	Roth (Karlsruhe, Germany)
Bovine serum albumin (for cell culture)	Invitrogen Corp (Paisley, Scotland, UK)
Chloroform	Sigma (St. Louis. MO, USA)
Collagenase	Worthington Biochemical Corp. (Lakewood, NJ, USA)
DATP	Roche (Mannheim, Germany)
DCTP	Roche (Mannheim, Germany)
DGTP	Roche (Mannheim, Germany)
Diaminobenzidine	Sigma (St. Louis. MO, USA)
Diethyl-dicarbonate	Sigma (St. Louis. MO, USA)
Dimethyl sulfoxide	Sigma (St. Louis. MO, USA)
Dithiothreitol	Sigma (St. Louis. MO, USA)
DNAse	Invitrogen Corp (Paisley, Scotland, UK)
dNTP Mix	MBI Fermentas (Vilnius, Lithouania)
DTTP	Roche (Mannheim, Germany)
Dulbecco's modified Eagle medium (DMEM)	Invitrogen Corp (Paisley, Scotland, UK)
Dulbecco's modified Eagle medium (DMEM) without Phenol Red	Sigma (St. Louis. MO, USA)
EDTA	MERCK (Darmstadt, Germany)
Entellan	MERCK (Darmstadt, Germany)
Epidermal growth factor	Sigma (St. Louis. MO, USA)
Ethidium bromide	Sigma (St. Louis. MO, USA)
Fetal calf serum	Gibco (Karlsruhe, Germany)
Formamide	Sigma (St. Louis. MO, USA)

Forskolin	Sigma (St. Louis. MO, USA)
Glucose	MERCK (Darmstadt, Germany)
Guanidine thiocyanate	Fluka Chemie AG (Buchs, Switzerland)
Hepes	Sigma (St. Louis. MO, USA)
Hexanucleotide Mix	Roche (Mannheim, Germany)
Hexanucleotide Mix	Roche (Mannheim, Germany)
Hyaluronidase	Sigma (St. Louis. MO, USA)
Hydrochloric acid	MERCK (Darmstadt, Germany)
Hydrogen peroxide	Roth (Karlsruhe, Germany)
Isoamylalcohol	MERCK (Darmstadt, Germany)
Isopropanol	Sigma (St. Louis. MO, USA)
Isopropanol	Sigma (St. Louis, Mo, USA)
KH2PO4	MERCK (Darmstadt, Germany)
Levamisole	Sigma (St. Louis. MO, USA)
L-Glutamine	Biochrom AG (Berlin, Germany)
Lipofectamine	Invitrogen Corp (Paisley, Scotland, UK)
Luciferin	Roche (Mannheim, Germany)
Magnesium chloride	MERCK (Darmstadt, Germany)
Marker 1kbPlus	Life Technologies (Paisley, Scotland, UK)
Octreotide	Novartis (Basel, Switzerland)
OPTIMEM 1	Invitrogen Corp (Paisley, Scotland, UK)
Paraformaldehyde	MERCK (Darmstadt, Germany)
PD 98059	Calbiochem (Darmstadt, Germany)
Penicillin+Streptavidine mix	Biochrom AG (Berlin, Germany)
Peptone	ICN Pharmaceuticals (Aurora, OH, USA)
Phenol	Roth (Karlsruhe, Germany)
Phosphate based buffer PBS (for cell culture)	Life Technologies (Paisley, Scotland, UK)
Photoemulsion	Ilford K5 (Eppelheim, Germany)
Plasmid Preparation Kit	QIAGEN (Hilden, Germany)
Polyacrylamide	Invitrogen Corp (Paisley, Scotland, UK)
poly-L-lysine-coated microscope slides (SuperFrost® Plus)	Menzel-Gläser (Braunschweig, Germany)
Potassium chloride (KCl)	MERCK (Darmstadt, Germany)
Reisin	Bio-Rad (Hercules, CA, USA)
Reporter lysis buffer	Promega GmbH (Mannheim, Germany)
Retinoic acid	Sigma (St. Louis, Mo, USA)
Reverse transcriptase (SuperScript II ™)	Invitrogen (Carlsbad, CA, USA)
Sarcosine, N-Lawryl-	Sigma (St. Louis, Mo, USA)

Sodium acetate dihydrate	MERCK (Darmstadt, Germany)
Sodium acetate trihydrate	MERCK (Darmstadt, Germany)
Sodium chloride (NaCl)	Roth (Karlsruhe, Germany)
Sodium dihydrogen phosphate mono-hydrate (NaH2PO4-H2O)	MERCK (Darmstadt, Germany)
Sodium hydrogen phosphate dihydrate (Na2HPO4.2H2O)	MERCK (Darmstadt, Germany)
Sodium peroxide (NaOH)	MERCK (Darmstadt, Germany)
Streptavidin	R&D systems (Minneapolis, MN, USA)
Taq DNA polymerase	MBI Fermentas
TEMED	Sigma (St. Louis, Mo, USA)
Terminal transferase	Roche (Mannheim, Germany)
Tissue-Tek®	Sakura Finetek Europe (Zoeterwoude, The Nederlands)
Toluidin Blue	Sigma (St. Louis, Mo, USA)
Triethanolamine	Sigma (St. Louis, Mo, USA)
Tris pure	ICN Pharmaceuticals (Aurora, OH, USA)
Trypsin	Sigma (St. Louis, Mo, USA)
Vector Red	Vector Laboratories (Burlingane, CA, USA)
WST-1 assay	Roche (Mannheim, Germany)
X-ray film	Kodak (New Haven, CT, USA)
Xylol	Roth (Karlsruhe, Germany)
Yeast extract powder	ICN Pharmaceuticals (Aurora, OH, USA)

Solutions

Collagenase Mix	1000 U/ml Collagenase : 400 mg/ 100ml solution Trypsin inhibitor: 10 mg/ 100ml solution Hyaluronidase : 100 mg/ 100ml solution BSA : 400 mg/ 100ml solution Dnase : 500 µl/ 100ml solution
Citric acid monohydrate (for antigen retrieval)	10mM Citric acid monohydrate: 2.1 g/L Adjust to pH 6.0
Formamide deionized	Add Reisin: 5ml/ 50ml Formamide
Formamide/4xSSC buffer	Formamide deionized: 50ml/ 100ml solution SSC 20x sterile : 20ml/ 100ml solution DEPC water : 30ml/ 100 ml solution
HDB buffer	Glucose : 18 mg/ 100ml solution Penicillin/Streptavidin : 1 ml/ 100ml solution Amphotericin B : 1 ml/ 100ml solution
LB medium	Peptone :10 g/L Yeast extract: 5 g/L NaCl : 5 g/L NaOH 1M : 2 ml/L Adjust to pH 7.0
Luciferase-assay buffer	Tris-HCl 1M pH 7.8 : 7.5 ml/ 100ml solution $MgCl_2$ 1M : 2.5 ml/ 100ml solution Before use add 4 µl/ml ATP 0.1 M
Paraformaldehyde (PFA)	4% paraformaldehyde :4 g/100 ml Sodium phosphate buffer : 20 ml/100ml solution Ampuwa water : 80 ml Add 1M NaOH to pH 7.4 Heat at 56°C to dissolve Filter and cool before usage Store at +4°C for maximum 2 days
Phosphate based buffer (PBS)	1x NaCl : 8 g/L KCl : 0.2 g/L $Na_2HPO_4.2H_2O$: 1.44 g/L KH_2PO_4 : 0.2 g/L Adjust to pH 7.4
Sodium acetate	2M

	Sodium acetate trihydrate: 27.2 g/ 100ml DEPC : 20 µl Add acetic acid to pH 4.0 Leave at room temperature overnight
Sodium phosphate buffer	50mM $Na_2HPO_4.2H_2O$: 7.06 g/L $NaH_2PO_4.H_2O$: 1.32 g/L Adjust to pH 7.4
Solution D	4M Guanidium thiocyanate 25 mM Sodium citrate pH 7.0 0.5% Sarcosyl dissolved in DEPC To complete the medium add: 180µl beta-mercaptoethanol/25ml solution
SSC	20x NaCl : 175 g/L Sodium citrate dihydrate: 88.23 g/L Adjust to pH 7.0 Filter and autoclave before use
Tris borate EDTA buffer (TBE)	10x Boric acid (H_3BO_3) : 61.83 g/L EDTA : 37.2 g/L Tris pure : 30.03 g/L Adjust to pH 8.0
Tris buffer	Tris pure: 12.114 g/L Adjust to pH 7.6
Tris-based buffer (TBS)	1x Tris pure : 2.42 g/L NaCl : 8 g/L Adjust to pH 7.6
Tris-HCl	1M Tris pure: 121.14 g/L Add 25% HCl to a pH 8.2

Tumor bank formation

In the time interval 1997-2001, 343 tumors were received from different neurosurgical departments in Germany and Italy. All the specimens were inserted in DMEM immediately after surgical excision and transferred to our laboratory as fast as possible. Tumors arriving within the same day or one day after the operation date, were processed for experiment as follows: after removing the fibers and debris, the tumor was divided into three portions; one part was destined for cell culture as will be described later in the Pituitary adenomas in primary cell culture session; the remaining piece was divided into two parts: one was put into an Eppendorf-tube for RNA extraction, and the other was snap-frozen with dry-ice, for morphological analysis.

The frozen piece was cut in in 8µm thick sections in cryostat and placed on sterile poly-L-lysine-coated slides. These slides were fixed in freshly prepared ice cold 4% PFA, dehydrated and stored in 96% ethanol at +4°C until usage. The first sections of each tumor were used for the determination of the pituitary hormonal content by immunohistochemistry. This is important in order, on one hand, to determine if there is any normal pituitary tissue neighboring to the tumor specimen and, on the other hand, to get some information about the histological type of the tumor. Some slides were also sent to a well known pathologist in the field (Prof. Dr. W. Saeger, Dept. of Pathology, Marienkrankenhaus, Hamburg, Germany) for a complete histological examination, which included hormonal content of the tumor, percentage of cells stained for a particular hormone, and proliferation index. The proliferation index was determined using the monoclonal antibody MIB-1 which binds to the proliferation-associated nuclear antigen Ki-67.

The next step was to prove the availability of the tumors for an immunohistochemical study. This was done, in part, with the immunohistochemistry for the intrapituitary

hormones. In the case of null cell adenomas, that do not stain for any hormone, *in situ* hybridization for alpha-subunit, FSH, and LH, was used to determine the suitability of the tumor, since this type of tumor is expressing at least one of these hormones at mRNA level despite the absence of any detectable protein. The two nuclear proteins Pit1 and SF1 were chosen to determine the suitability of acromegaly-associated adenomas and prolactinomas, and non-functioning pituitary adenomas respectively, for immunohistochemical analysis of the nuclear protein in question.

The piece destined for RNA extraction had to be proven that is containing only tumor cells and no normal pituitary contamination. In part, immunohistochemical analysis on tissue cuts, adjacent to the piece destined for the RNA extraction, could provide information about any normal pituitary cells present in this part of the tumor. However, to be absolutely sure, PCR was performed for the transcription factors SF1 and Pit1 (Zafar et al., 1995). By this way, prolactinomas and acromegaly-associated adenomas were excluded from any PCR study if they were shown to express SF1 and non-functioning adenomas were excluded if they expressed Pit1.

Cell culture

Cell lines

The immortalized cell lines used in this study were the rat mammosomatotroph cell line GH3 and the mouse corticotrophinoma cell line AtT-20. Cells were grown in DMEM supplemented with 10% FCS, 2.4g/L Glutamine, 2.5 ng/L Amphotericin B, and 10^5 U/L Penicillin/ Streptomycin, and incubated in humid atmosphere with 5%CO_2 at 37°C. When confluent, cells were washed with PBS, trypsinized, centrifuged at 1200g for 4 min, and platted according to the demands of each experiment.

Pituitary adenomas in primary cell culture

The piece from pituitary adenoma, destinated for cell culture, was washed throughout with HDB buffer and was mechanically dispersed into small fragments. These fragments were inserted in solution containing 4g/L collagenase, 0.01 g/L DNAse II, 0.1 g/L soybean trypsin inhibitor and 1 g/L hyaluronidase II and were enzymatically dispersed in 37°C for 30-45 min. Cell viability was determined by acrinide orange /ethidium bromide staining. Cells were diluted with DMEM, supplemented with 10% FCS, to a density of $2x10^5$ cells/ml, distributed in 48-well tissue culture plates, and maintained at 37°C for 3-4 days before being treated and assayed.

Agent stimulation experiments

Before each stimulation experiment, cells were serum-deprived for 24 hrs in order to arrest in the G0 phase. In general, stimulants were dissolved in serum free medium, to avoid possible interactions with growth factors present in the serum. Unless otherwise indicated, duration of each stimulation was 24 hours. After this time, medium was removed, cells were washed with PBS, and Solution D was added for RNA extraction.

For stimulation experiments involving retinoic acid, phenol free DMEM was used. During incubation with retinoic acid, the plate, containing the cells, was wrapped in aluminum foil since this substance is light sensitive.

Cell proliferation and viability

Cell proliferation was measured using the WST-1 proliferation assay (Roche Molecular Biochemicals, Penzberg, Germany). This assay measures the activity of the mitochondrial succinate-tetrazolium reductase system. In living cells, the WST-1

substance is converted to a dye which can be read by an ELISA reader. For this assay, cells were seeded in a 96-well plate (1000 cells/ well), and after 3-4 days they were stimulated. After 24 hours, the WST-1 reagent was added to a final dilution 1:10, and after 2 hours incubation, the plate was measured with the ELISA reader at 450 nm.

Cell viability was determined by acridine orange/ ethidium bromide staining. Acridine orange, but not ethidium bromide, can be incorporated into the cells. Therefore if the cell membrane is intact, i.e. the cell is alive, only acridine orange will pass through the cell membrane and the cell nucleus will stain green; if the cell is dead the ethidium bromide will reach the nucleus bypassing the destroyed cell membrane, intercalate into the DNA and stain the cell nucleus red. On a Neubauer chamber, a mix of equal volumes of cell suspension and 1:1 acridine orange/ethidium bromide was placed. Examination was done under UV light. The cell viability was determined as the percentage of green (= alive cells) in the total number of cells seen on each chamber area.

Gene expression studies

RNA extraction

Normal and adenomatous pituitary was embedded in 800 µl Solution D and homogenized using the Ultra-TURRAX T8 (IKA Labortechnik) homogenizer. Solution D is composed by guanidine thiocyanate and beta-mercaptoethanol, which by inhibiting the Rnases, activated after cell death, prevents possible RNA degradation. To the homogenized tissue, 80 µl (1/10 of the initial Solution D volume) sodium acetate was added to precipitate the genomic DNA, followed by 800 µl phenol and 160 µl Chloroform: Isoamylalcohol (49:1). After 15 min incubation in ice, the solution was centrifuged for 30 min at maximum speed (12000 rpm) at 4°C. At this stage, due

to phenol, two phases are clearly visible, which are separated by a thin zone containing the precipitated genomic DNA. The lower phase contains the phenol-soluble proteins and cell remains. The upper phase, which is containing the RNA, was carefully collected, taking special care not to be mixed with the genomic DNA-containing intermediate zone, and transferred to a new Eppendorf tube. Isopropanol was added in a volume equal to the upper phase, and the solution was incubated in –20°C for 2-12 hours to allow the complete RNA precipitation. After 30 min centrifugation, the supernatant was discarded, the pellet was washed with ice-cold 70% ethanol, and the solution was centrifuged for another 30 min. Then the ethanol was carefully discarded, the pellet was let to completely air dry, before being dissolved in 20-100 µl water supplemented with 0.1% (v/v) DEPC. DEPC inactivates RNases, thus protecting RNA against degradation.

RNA from cell culture was extracted as described above. After removing the cell culture medium and washing with PBS an appropriate amount of solution D was added in the petri-dish or well (usually it is 1 ml solution D per petri-dish, 800 µl per well in a 6-well plate and 200 µl per well in a 48-well plate).

When enough material, each RNA sample was aliquoted, keeping one aliquot in -20°C for current use, and the remaining at -80°C for long term storage.

The concentration and cleanness of each RNA sample was determined using a spectrophotometer (Ultrospec II, Pharmacia). Nucleic acids are having an absorption maximum at 260 nm, while for proteins the value reaches the 280 nm. The ratio A_{260}/A_{280} gives information about the quality of the RNA preparation, i.e. if it is free from proteins and phenol rests. The optimal value for a very clean RNA solution lies between 1.9 and 2.0. RNA concentration is calculated using the following formula: $A_{260} \cdot 40 \cdot 60 = \chi$ µg/µl, where 40 is the concentration in µg/µl of RNA giving A_{260}

value equal to 1, and 60 is the dilution in which the RNA is measured (1µl RNA solution + 59µl DEPC water)

Reverse transcriptase – polymerase chain reaction (RT-PCR)

1 µg of RNA was incubated with 2mM dNTP mix, 62.5 A_{260} U/ml random primers (Hexanucleotide Mix), 10mM dithiothreitol (DTT) and 200 U reverse transcriptase (SuperScript II) all diluted in 1x First Strand buffer, at 45°C for 1 hour. The reaction is inactivated by boiling at 95°C for 5 min. The cDNA produced was stored at -20°C.

For the PCR reaction 2 µl cDNA were added in a reaction mixture containing:

3 µl 10x PCR buffer

1.8 µl 25mM $MgCl_2$

3 µl 2mM dNTP Mix

1 µl amplification primer 1; 10 pmol/µl

1 µl amplification primer 2; 10 pmol/µl

0.3 µl *Thermus aquaticus* (*Taq*) DNA polymerase

17.9 µl autoclaved, distilled water

The PCR reaction parameters were: denaturation at 95°C for 1 min, annealing at 55-65°C for 1 min, polymerization at 72°C for 1 min, for 35-40 cycles. In every PCR reaction, cDNA from normal human pituitary was used as positive control and reaction in absence of template as negative control. To ensure that the RNA samples were not contaminated by genomic DNA, PCR reaction was carried for a housekeeping gene (beta-actin or GAPDH) using as template RNA sample which had not been reverse-transcribed.

After each reaction, the products were separated in a 1.2-1.5% agarose gel depending on the size of the product (1.2% for 400-1000 bp, 1.5% for 200-400 bp) and visualized by ethidium bromide under UV light. Electrophoresis took place in 1x TBE buffer for 15-20 min at 80 V. The 1kb Plus DNA marker was used to determine the fragment size.

The primers used are listed in Table 1, with their sequences, annealing temperature and predicted product size. The sequence of each was checked using the NCBI BLAST program, to exclude the possibility of a sequence similarity with genes other than the one under investigation. All primers were synthesized by MWG Biotech, were reconstituted with autoclaved distilled water to reach a concentration of 100 μM, and were stored at -20°C. The annealing temperature for each pair of primers was determined by PCR in a range of 55, 60, and 65°C using cDNA from normal human pituitary as template. The optimum temperature was the one which was yielding an intense signal with no secondary amplification fragments.

Nested-PCR

To determine the expression of EGFr and EGFr variant III, nested PCR was performed as previously described (Schlegel et al., 1994). In brief, PCR was performed on 2μl from the reverse-transcription reaction, using NP1/2 as the outer primer pair and NP6/7 or JS3/4 as inner primer pairs. NP6/7 amplifies a 1002 bp PCR fragment in the case of EGFr wild type or a 201 bp fragment remaining from the deletion of the coding region of exon 2 through exon 7 that characterizes EGFrvIII. Control PCR using JS3/4 reveals a 391 bp band corresponding to EGFr wild type, which is expected in all samples expressing EGFr or EGFrvIII.

Semiquantitative RT-PCR

Semiquantitative PCR was performed in order to quantify the levels of ZAC gene expression in pituitary adenomas. Two µl cDNA were amplified with the same PCR reagents as described above but, instead of using dNTP mix, each dNTP was added separately as follows:

1.2 µl dTTP 10mM

1.2 µl dCTP 10mM

1.2 µl dGTP 10mM

0.2 µl dATP 10mM

plus 0.5 µl (5µCi) α^{33}P-dATP 4mM.

Although the time and temperature were used as defined for the simple PCR, the number of cycles was 25 for ZAC and 20 for beta-actin. PCR products were electrophoresed in a 6% acrylamide gel, which was then dried (Model 583 Gel dryer, Bio-Rad) and exposed to X-ray film (Kodak) or to Phospho-Imager. OD for ZAC and beta-actin were quantified by digital analysis (Tina 4.0, Raytest, Munich, Germany). After subtraction of background signals the relative gene expression levels was determined as the ratio of OD gene /OD beta-actin. PCR reactions were performed in triplicates for each sample.

In situ hybridization

ISH was performed to study the distribution of ZAC mRNA expression in human normal and adenomatous pituitary. Eight oligonucleotides (ODNs) were designed at nucleotide positions 47-92, 300-344, 525-569, 614-659, 777-822, 875-920, 907-952, and 1002-1046 (Fig.6) and synthesized by Amersham-Pharmacia (Freiburg, Germany). The ODNs were labeled at the 3'-end with [α-^{33}P]dATP by terminal transferase.

Already fixed tissue slides were rehydrated in PBS, pretreated with triethanolamine, to facilitate the better access of the ODNs into the cell nucleus, dehydrated, and left to completely dry out. ODNs were dissolved in hybridization buffer and applied on the dried tissue. A mix of labeled ODNs together with a 100-fold excess of unlabeled ODNs was always applied as a control. The tissues were well covered by paraffin films and the slides were placed in a box wet with 50% Formamid/4xSSC buffer, which was then well shielded and placed in a 42°C dry oven. After 16-24 hours incubation, sections were well washed to remove every excess ODNs, dehydrated, and left to dry. A quick image of the hybridization was obtained by exposing the sections to a phosphorimager analyzer. Then the slides were dipped in Ilford K5 photoemulsion for 28 days, developed, and counterstained with toluidine blue.

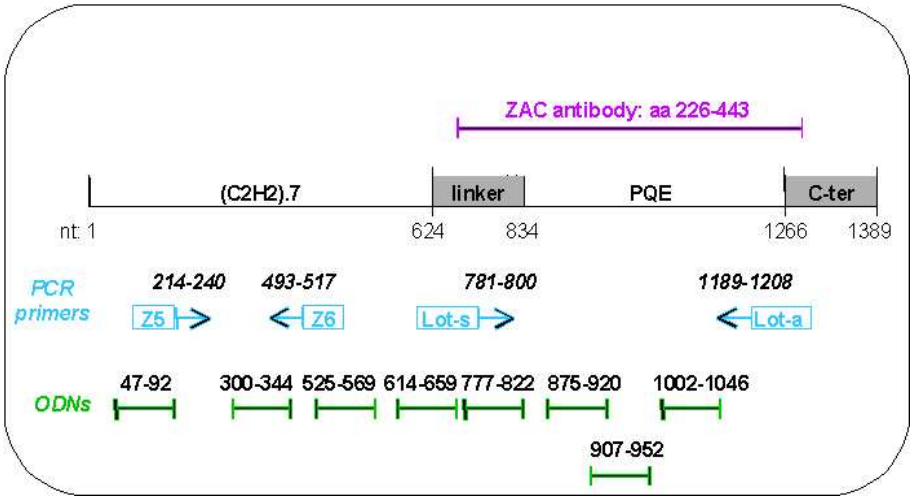

Fig.6. Schematic presentation of the relative positions of the primer and ODN sequences on ZAC. (C2H2).7: zinc finger region; linker: linker region; PQE: proline/ glutamate/ glutamine rich region; C-ter: COOH terminus; aa: aminoacids against which the ZAC antibody is raised.

Name	Sequence (5'-3')	Tm	Fragment (bp)
beta-actin-s human	ACG GGG TCA CCC ACA CTG TGC	60	660
beta-actin-a human	CTA GAA GCA TTT GCG GTG GAC GAT G		
COUP-s	GT CCC TGC TAC GCC TCA CC	65	440
COUP-a	AGA GTT TCG ATG GGG GTT TT		
GAPDH-s rat	ATG GTG AAG GTC GGT GTG AAC G	60	495
GAPDH-a rat	GTT GTC ATG GAT GAC CTT GGC		
Lot-s	CAT AGC CTC ACC CTC AGT CC	60	408
Lot-a	AGA GTG CTA TTC CCA AAG GT		
MEN1-s	GCT GGC TGT ACC TGA AAG GA	60	275
MEN1-a	CTT GTG GTA GAG GGT GAG TG		
NP1	GAG CCG GAG CTC TTC GGG GA	45	-
NP2	CAG TTC CTG TGG ATC CAG AAG AGG		
NP6	CGG CGA GTC GGG CTC TGG AGG	60	1002 (EGFrWT) or
NP7	GTA GCA TTT ATG GAG AGT GAG		201 (EGFrvIII)
SF1-s	GCA TCT TGG GCT GCC TGC AG	71	230
SF1-a	CCT TGC CGT GCT GGA CCT GG		
Pit-s	AGT GCT GCC GAG TGT CTA CCA	59	560
Pit-a	TTT CTT TTC CTT TCA TTT GCT		
JS3	GTA CTA CGA GAA TTC CTA ATG CC	60	391
JS4	CTG TGC AGC CTG CAG CAC ACT GGT		
Z5 (ZAC-s)	AAC CGG AAA GAC CAC CTG AAA AAC CAC	60	304
Z6 (ZAC-a)	GTC GCA CAT CCT TCC GGG TGT AGA		

Table.1. List of primers used. *1st column*: name of primer. NP1, NP2, NP6, NP7, JS3 and JS4 primers were used for the nested PCR for the identification of EGFr (*EGFrWT*) and EGFr variant III (*EGFrvIII*). *2nd column*: Sequences; *3rd column*: annealing temperature; *4th column*: Size of PCR product. When using the NP6 and NP7 set of primer, two type of products can be expected: a 1002 bp fragment corresponding to the wild type of EGFr or a 201 bp fragment corresponding to the EGFrvIII.

Protein studies

Immunohistochemistry

Principle

In order to determine the expression of a gene at protein level and to visualize the protein distribution and intensity of expression in the normal cellular and tissue environment, the method of immunohistochemistry was employed. Specific antibodies (*primary antibodies*) are used to identify the protein in question. This antigen-antibody binding is followed by a series of immunological interactions, which lead to the enzymatic reaction that will allow the visualization of the signal. As shown schematically in Fig.7A, the primary antibody already attached to its epitope is linked to a secondary antibody, that can recognize and therefore bind to the epitopes of the host animal in which the primary antibody was raised; i.e. when the primary antibody is mouse monoclonal the secondary antibody is anti-mouse and so on. The secondary antibody is then linked to the enzymatic complex that will catalyze the chromogen used and visualize the signal. One system used here is the avidin-biotin system, in which the secondary antibody is conjugated to biotin beads, which can bind to the avidin-enzyme complex. The enzyme can be peroxidase or alkaline phosphatase.

Another enzyme used is the alkaline phosphatase anti-alkaline phosphatase complex, which can be produced in mouse or rabbit, and therefore be recognized by the anti-mouse or anti-rabbit IgG, used as secondary antibody (Fig.7B).

Primary antibodies

The primary antibodies and dilutions used are given in Table 2. Each antibody was tested and optimized on control tissue, which was usually human normal pituitary gland. Other control tissues used were human tonsils, for antibodies associated with

cell cycle and proliferation, and meningiomas and glioblastomas, for the antibodies against EGFr. The standard testing dilution range was 1:50 to 1:2000.

Antibody	Source	Host	Dilution
EGFr (F 4)	Sigma (St. Louis. MO, USA)	mouse	1:100
EGFr (E 30)	Biogenex (San Ramon, CA, USA)	mouse	1:20
Menin	Gift from Dr V. Ciminale (Univ. of Padova, Italy)	mouse	1:100
Human Ki-67/MIB1	DAKO (Glostrup, Denmark)	mouse	1:100
Pit1	Santa Cruz Biotech (Santa Cruz, CA, USA)	rabbit	1:100
SF1	Upstate Biotech (Lake Placid, NY, USA)	rabbit	1:300
S100	Biogenesis (Poole, UK)	rabbit	1:20
Human TSH	Immunotech (Marseille, France)	mouse	1:500-800
Human FSH	Immunotech (Marseille, France)	mouse	1:500-800
Human LH	Immunotech (Marseille, France)	mouse	1:500-800
Human Alpha-Sub	Immunotech (Marseille, France)	mouse	1:500
Human PRL	Immunotech (Marseille, France)	mouse	1:400
Human ACTH	DAKO (Glostrup, Denmark)	mouse	1:100
Human GH	Gift from Dr C. J. Strasburger (Medizinische Klinik, Munich, Germany)	mouse	1:800
COUP-TFI (T-19)	Santa Cruz Biotech (Santa Cruz, CA, USA)	goat	1:500
ZAC	Gift from Dr L. Journot (CNRS, Montpellier, France)	rabbit	1:800

Table.2. List of the primary antibodies used in this group of studies. Antibodies made in mouse are monoclonal, while the ones made in rabbit and goat are polyclonal.

Protocol for cryostat sections

Frozen normal and adenomatous pituitary tissues were cut in a cryostat (Leica CM3050 S), in 8 µm thick sections. The slides were fixed in 4% freshly prepared ice cold PFA, dehydrated and stored in 96% ethanol, at +4°C. At the time of the experiments, sections were briefly incubated in TBS for 5 min, followed by 30 min

blocking. Blocking was performed using serum from the same animal species to the one that gave rise to the secondary antibody; in our case blocking solutions were consisting of horse or goat serum diluted 1:10 in TBS. After blocking the endogenous peroxidase activity with 1% H_2O_2 diluted in TBS for 15 min, sections were incubated overnight with the primary antibody at 4°C. Then, after washing three times with TBS, 5 min each, sections were incubated for 30 min with the secondary antibody at room temperature, and after 3 more washings, with the ABC complex for another 30 min. The ABC was prepared at least 30 min prior usage, in order to give time for the complex to form, in saline-free tris-based buffer (Tris buffer). After washing three times in TBS, slides were immersed in freshly prepared DAB (1 mg/ml) supplemented with 0.01% hydrogen peroxide. Because DAB is light-sensitive, the reaction was carried on in semi-darkness, and the reagent was covered with aluminum foil during the experiment. The time of incubation in DAB varied and was determined for each primary antibody separately. Optimal time was the one which was giving the strongest expected signal with the lowest possible background.

A variation of the method made use of ABC linked to alkaline phosphatase (ABC-AP) and in that case, color was developed using the Vector Red kit. Levamisole (10 mM) was added to the reaction mixture to block the endogenous alkaline phosphatase activity. This method was used to immunohistochemicaly determine the hormonal content for each tumor.

After the development of a satisfactory signal, slides were washed many times in TBS and counterstained with toluidine-blue, which is staining the cell nuclei pale blue allowing an easy view of the tissue organization. Excess color was removed by immersing the slides in 70% ethanol supplemented with acetic acid, and sections were dehydrated, fixed in xylol and coverslipped using Entellan. Evaluation of immunohistochemistry was done using the Axioscop II microscope (Zeiss).

In the ZAC study, immunoreactivity for each tumor was quantified as follows: Staining was classified as: absent “0”, weak “1”, moderate “2” and strong “3” and the percentage of cells in each category was determined. Intensity of immunoreactivity was calculated: 0 x % of unstained cells + 1 x % of weakly stained cells + 2 x % of moderately stained cells + 3 x % of strongly stained cells. Each value was divided by 300 - a hypothetical maximum for 100% of the cells being strongly stained - providing final values between 0 (no immunoreactivity) and 1 (maximum immunoreactivity).

In the Menin study, intensity of the nuclear staining was scored as follows: weak “+”, medium “++”, strong “+++”.

Protocol for paraffin sections

Paraffin sections were deparaffinized according to the following protocol:

5 min xylol

5 min xylol

5 min 100% ethanol

5 min 100% ethanol

3 min 96% ethanol

3 min 80% ethanol

3 min 70% ethanol

3 min 50% ethanol

5 min distilled water

When a nuclear protein was to be detected antigen retrieval was performed at this stage by microwaving as follows: after washing in distilled water for 1 min, sections were transferred in citric acid monohydrate buffer, 10mM, pH 6.0, and microwaved for 5 min at 750 W. Then they were allowed to cool at room temperature for 1 min before microwaving once more for 4.5 min. This last step was repeated once more.

After the last microwaving they were left at room temperature for 20 min to completely cool down. Then they were transferred in distilled water and washed 3 times, 2 min each, before being transferred in TBS and processed according to the appropriate protocol.

Double immunohistochemistry

In order to see whether two proteins are co-expressed within a cell, double immunohistochemistry was used. Extra care was needed in the design of the protocol in order to avoid cross-links between the different antibodies and subsequently false positive results. In most of the cases, antibodies against the two proteins in question were one monoclonal and one polyclonal; therefore, they were processed in parallel as follows: after washing in TBS and blocking in goat serum, sections were incubated with both antibodies at 4°C overnight. The following day, after washing three times in TBS, sections were incubated in a solution containing goat anti-rabbit biotinylated Ig and goat anti-mouse IgG for 30 min. The procedure was repeated once more, and then sections were incubated in a mix of ABC and mouse alkaline phosphatase anti-alkaline phosphatase (APAAP) for 30 min, twice. After the second round was completed the color was developed for the polyclonal antibody using DAB, and after several washing with TBS, the second color was developed using Vector Red. Sections were incubated in Vector Red for 40 min twice. Because the anti-mouse IgG – APAAP method lacks the signal amplifying properties of the avidin-biotin procedure there is need to repeat each step two times and to prolong the time of color development sometimes to two hours. In addition, in some cases, the dilution of the primary antibody had to decrease when used with the anti-mouse IgG – APAAP protocol.

When both primary antibodies were monoclonal, immunohistochemistry for the one protein was performed and completed before the immunohistochemistry for the other one. It was proven that performing the immunohistochemistry, which is visualized with DAB prior to the one using Vector Red, yields to better signal with much less background.

The anti-mouse IgG – APAAP method was usually used for membrane/ cytoplasmatic proteins, for which there was evidence of being highly expressed. For nuclear proteins, this type of staining was not giving good results because the red color was masked by the Tholuidin-blue used in the counterstaining.

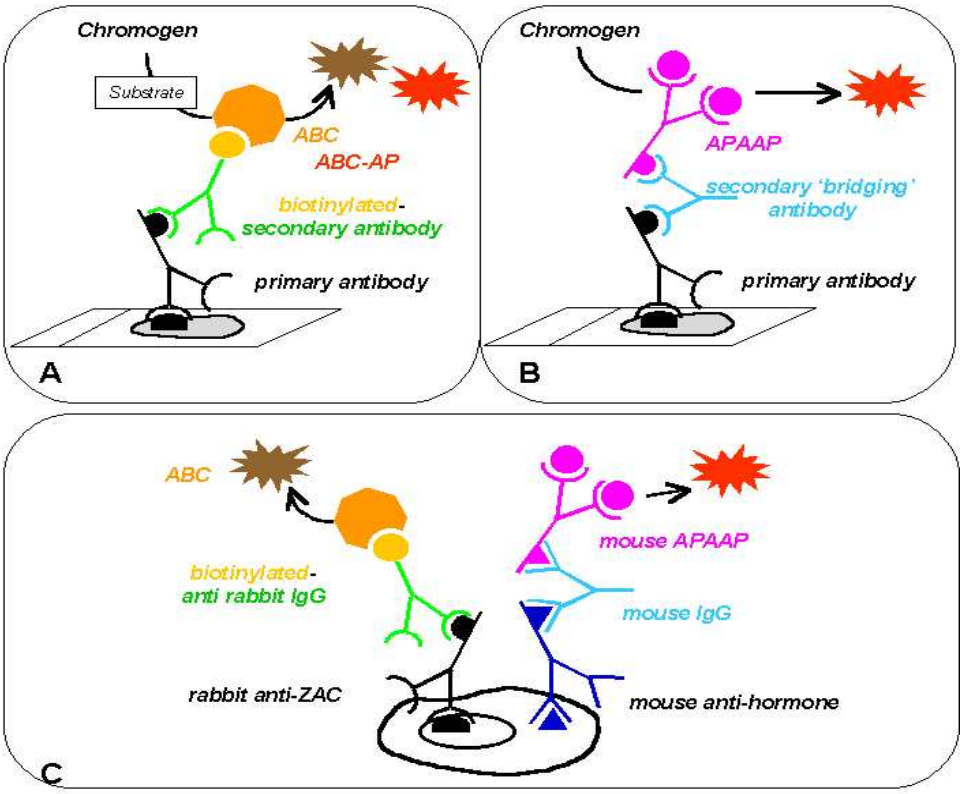

Fig.7. Simplified diagrams showing the principle of the immunohistochemical techniques used. *A.* Single immunohistochemistry using the avidin biotin amplification system. The primary antibody binds to the tissue antigen of interest and a biotinylated secondary antibody binds to an epitope of the primary antibody. The biotin labeling forms a complex with the avidin-biotin complex (ABC). The ABC contains a peroxidase which catalyses a reaction using hydrogen peroxide as substrate and DAB as chromogen. At the end of the reaction the chromogen gives a brown color to the sites where the

antibody has bound. An alternative is to use ABC linked to an alkaline phosphatase (ABC-AP), which produces red color. *B*. Single immunohistochemistry using alkaline phosphatase. The secondary antibody acts as a bridge between the primary antibody and the alkaline phosphatase anti-alkaline phosphatase (APAAP) complex, by recognizing epitopes in the primary and anti-alkaline phosphatase antibodies. The chromogen is Vector Red and gives red color to the immunoreactive sites. Because there is no amplification system used here the reaction yields faint signals and is suitable only in case of of proteins, which are suspected to be highly expressed. *C*. Double immunohistochemistry to determine in which endocrine cells is the protein under question (e.g. ZAC) expressed. For the detection of ZAC the ABC system is used, while for the detection of the hormone the APAAP system is employed. Note that the primary antibodies are derived from different animals, and the system is build up in such way that there is no probability of cross reaction between the antibodies used for each detection.

Transfection studies

Plasmid preparation

The plasmids used in this study were the COUP-TFI expression vector, which expresses 1.5Kb mouse COUP-TFI cDNA under the control of the cytomegalovirus (CMV) promoter; the empty vector, which was used as a control, and a vector expressing the luciferase gene under the control of 770 bp of the rat POMC promoter.

Competent bacteria, i.e. bacteria pretreated in such way that they posses pores and can therefore easily incorporate a plasmid, were transformed, left to grow overnight at 37°C on agar containing ampicillin, and stored at +4°C. A colony was picked from each plate and left in 250ml LB medium containing 50µg/µl ampicillin overnight at 37°C. Plasmid preparation was performed using the QIAGEN plasmid purification system.

To confirm that the correct plasmid is going to be used for the transfection experiment, 5µl of each plasmid were digested with EcoRI and HindIII restriction

enzymes. When run the products in 1.2% agarose gel COUP-TFI was present as the predicted two bands and the empty vector as one.

Cell transfection

Cell transfection was performed on confluent AtT-20 cells grown in a 6-well plate, using Lipofectamine as follows: 500ng of POMC-Luc and COUP-TFI or empty vector plasmids were mixed with 8µl/well of Lipofectamine in OPTIMEM1 medium, and incubated at room temperature for 45 min; during this time, the Lipofectamine neutralizes the plasmid DNA and surrounds it by lipid molecules. Cells were washed two times with OPTIMEM1 and transfected with plasmid for 6 hours. At this step the lipid molecules of the lipofectamine will fuse with the cell membrane, emptying the plasmid content into the cytoplasm. Then the transfected cells were supplemented with fresh DMEM containing 10% FCS and left for 18 hours to recover before stimulating with retinoic acid for 6 or 24 hours.

Reporter assay

To determine the levels of POMC driven luciferase expression after stimulation with retinoic acid, a luciferase reporter assay was employed. Luciferase is an enzyme found in the *Photinus pyrallis*, the common firefly. Luciferase emits light in the presence of luciferin and ATP, which can be detected at 562nm wavelength.

After washing one time with PBS, cells were lysed using the reporter lysis buffer and scraped. The lysates were transferred into Eppendorf tubes and briefly centrifuged. The supernatants were collected to be assayed as follows: 50µl supernatant was mixed with equal amount of ATP-containing Luciferase-assay buffer, and luciferase activity was measured in a Berthold luminometer, using 4 mg/ml luciferin as substrate. Experiments were carried out in triplicates.

Statistics

For the quantification of ZAC immunoreactivity, only the tumor groups with sample sizes ≥7 were considered (acromegaly-associated pituitary adenomas, prolactinomas, Cushing's adenomas, gonadotrophinomas, and null cell adenomas). The means of the final values for the ZAC immunoreactivity within these groups were compared by a one-way analysis of variance (ANOVA), followed by pairwise comparisons using Scheffé's post hoc test in the case of significant group effects. To approach normality and homogeneity in the data, the final values for the ZAC immunoreactivity were first transformed with the "arcsin" transformation and then used in the ANOVA. Associations between final values for ZAC immunoreactivity and proliferation index or grade of invasiveness for each tumor were investigated by using the Spearman correlation coefficient. As nominal level of significance $\alpha < 0.05$ was accepted. It was reduced (adjusted according to Bonferroni procedure) for all post hoc tests in order to keep the type I error ≤ to 0.05.

To determine the significance of octreotide's effect on cell proliferation, the means of the OD 450 values were compared by one-way ANOVA.

Statistics to determine the significance of retinoic acid treatment on POMC promoter activity, were performed using one-way ANOVA in combination with the Scheffé's test.

RESULTS ON
ZAC AND ITS REGULATION IN PITUITARY ADENOMAS

ZAC expression in human normal pituitary gland

Examination of 7 human normal pituitary glands by ISH revealed high levels of ZAC mRNA expression in the adenohypophysis and low in the neurohypophysis (Fig.8 A). Abundance of silver grains was observed in all the endocrine cells of the anterior lobe (Fig.8 C). There was almost no sign of silver grains in the presence of excess of unlabeled oligodeoxynucleotides used as control of specificity (Fig.8 B and D).

A rabbit polyclonal antibody generated against the ZAC C-terminal (Fig.6; Pagotto et al., 2000) was used to assess the expression of ZAC protein using immuno-histochemistry. Strong immunoreactivity for the ZAC protein was evident in the nuclei of all the endocrine cells (Fig.8 E), which is in agreement with the nuclear localization of ZAC as previously described (Varrault et al., 1998). Signal specificity was demonstrated after incubating an adjacent section with the pre-immune serum (Fig F). Double immunohistochemistry with all the anterior pituitary hormones, revealed that all hormone producing cells have intense immunoreactivity for ZAC, indicating that ZAC expression is not restricted to one cell type (examples of colocalization are shown in Fig.8 G and H).

The expression profile of the protein, as detected by immunohistochemistry supports the findings by *in situ* hybridization, indicating that ZAC is abundantly expressed in the human anterior pituitary and is present in all endocrine adenohypophyseal cells.

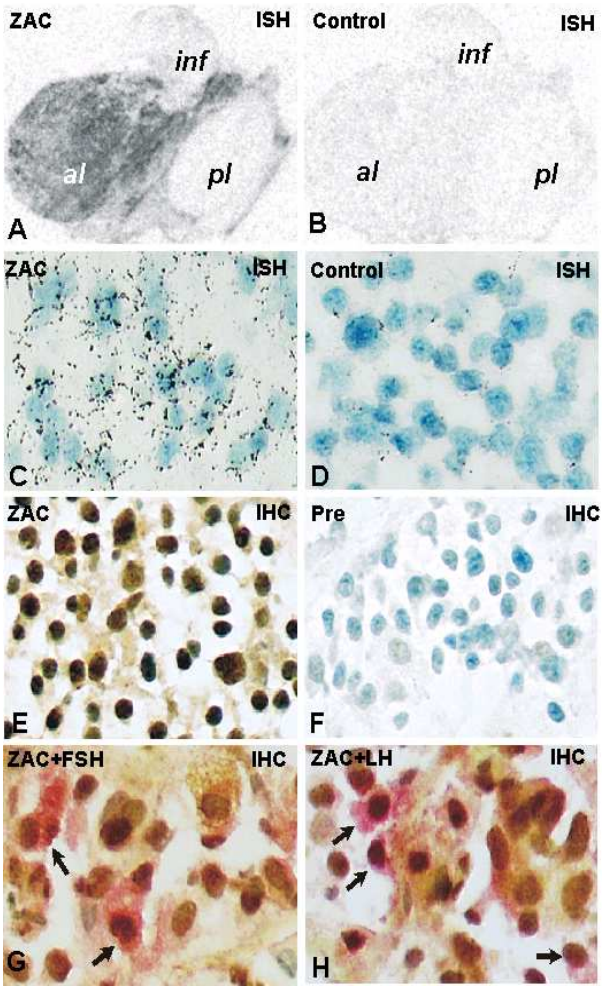

Fig.8. ZAC mRNA and protein expression in human normal pituitary. *A.* ZAC mRNA expression, as determined by ISH, is high in the anterior lobe of the pituitary (*al*), and faint in the infundibulum (*inf*) and posterior lobe (*pl*). *C.* ZAC mRNA is abundant (*silver grains*) in all cells in the anterior pituitary lobe. *B* and *D.* Control for signal specificity using an excess of unlabeled oligonucleotides. *E.* Strong immunoreactivity for ZAC protein (*brown*) is detected in the nuclei of all adenohypophyseal cells. *F.* Specificity of immunoreactivity demonstrated using preabsorbed serum. *G* and *H.* ZAC colocalization with FSH (*G*) and LH (*H*) (black arrows) in normal anterior pituitary. All sections are counterstained with toluidine blue.

ZAC gene expression in pituitary adenomas.

The levels of ZAC expression were assessed in 25 pituitary adenomas (Fig.9), by semi-quantitative PCR using two different sets of primers (Fig.6). All the pituitary adenomas had lower levels of expression when compared to the normal pituitary gland. However, ZAC mRNA levels were dramatically reduced in the gonadotrophinomas and were absent in most null cell adenomas (Fig.9).

Tumor No.	Clinical Diagnosis	Immunohistochemical Analysis
1	ACRO	GH
2	ACRO	GH
3	ACRO	GH
4	ACRO	GH/PRL
5	ACRO	GH/PRL
6	PROL	PRL
7	PROL	PRL
8	TSH	TSH
9	CUSH	ACTH
10	CUSH	ACTH
11	CUSH	ACTH
12	GONA	FSH
13	GONA	FSH/LH
14	GONA	FSH/Sub
15	GONA	Sub
16	GONA	FSH/LH
17	GONA	FSH/LH
18	GONA	FSH
19	NULL	None
20	NULL	None
21	NULL	None
22	NULL	None
23	NULL	None
24	NULL	None
25	NULL	None

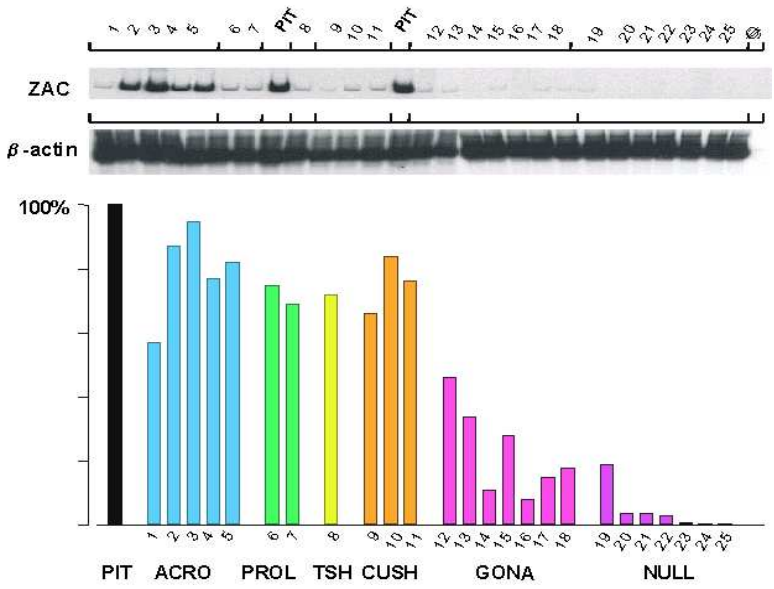

Fig.9. ZAC gene expression in 2 normal pituitaries and 25 pituitary adenomas as determined by RT-PCR. The numbers are corresponding to the tumors shown in the table; PIT: normal pituitary; Ø: PCR in absence of template. The graph shows quantitative analysis of the absorbance A_{ZAC}:$A_{\beta\text{-actin}}$ ratio in the samples shown in the ZAC and β-actin gel photos. The mean of ZAC:b-actin ratios is set as 100% of seven normal pituitaries. The values of the pituitary adenomas are presented as a percentage of the normal pituitaries. ACRO: acromegaly associated adenomas; PROL: prolactinomas; TSH: thyrotrophinomas; CUSH: Cushing's associated adenomas; GONA: gonadotrophinomas; NULL: null cell adenomas. ZAC gene expression is reduced in most adenomas when compared to the PIT, but the reduction is dramatic and the signal is many times lost in the GONA and NULL.

Study of 65 cases (14 acromegaly associated adenomas, 8 prolactinomas, 7 Cushing's adenomas, 2 thyrotrophinomas, 19 gonadotrophinomas, and 15 null cell adenomas) by *in situ* hybridization using eight different oligodeoxynucleotides (their binding sites is indicated in Fig.6) confirmed these findings. No or only weak signal was present in the vast majority of non-functioning pituitary adenomas (Fig.10).

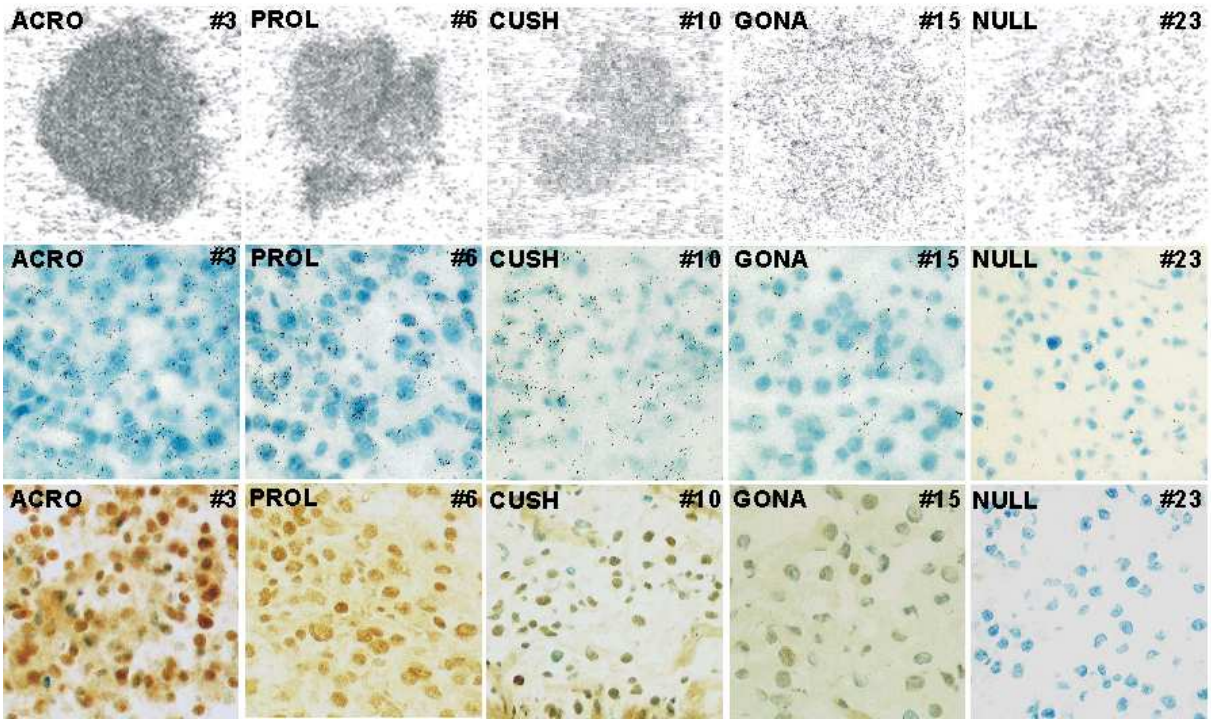

Fig.10. Expression of ZAC in a representative member of each adenoma type, as determined by ISH and IHC. In the top row are displayed phosphorimages showing ZAC mRNA expression, in the middle row, histoautoradiographs of the same sections, and in the bottom row, ZAC immunoreactivity in adjacent sections of the same adenomas. ZAC mRNA and protein levels are high in ACRO, PROL and CUSH but are dramatically reduced in GONADO and lost in NULL (20x magnification).

ZAC protein levels in pituitary adenomas

Immunohistochemical analysis of the same set of tumor confirmed the findings at mRNA level. ZAC protein staining was moderate in most pituitary adenomas when compared to normal pituitary, but was faint or absent in non-functioning pituitary adenomas (Fig.10). Quantification of the signal intensity and distribution revealed significantly less intense signal in both gonadotrophinomas and null cell adenomas, when compared to the other tumor subclasses. In the cases of non-functioning tumors displaying ZAC immunoreactivity, the signal was heterogeneously distributed, suggesting that areas immunopositive for ZAC can be restricted to areas

immunopositive for hormones. However, using double immunohistochemistry it became clear that ZAC staining is independent of the hormonal status of the cells, being present or absent in gonadotrophin-producing cells and gonadotrophin-negative areas/cells.

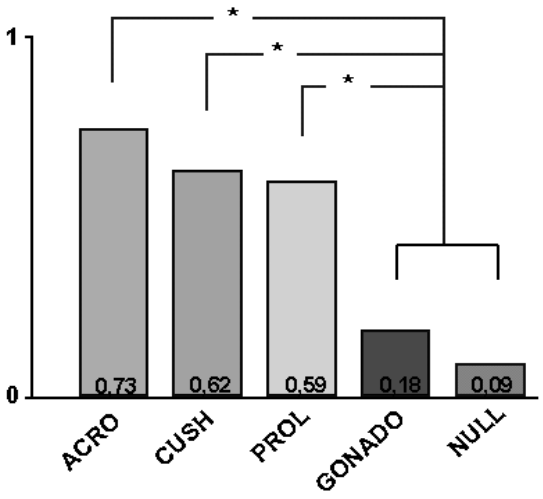

Fig.11. Quantitative presentation of ZAC immunoreactivity. The intensity of ZAC signal was calculated in a total of 65 pituitary adenomas. The mean values in GONA and NULL are significantly lower compared to those of ACRO, PROL and CUSH (Scheffe's *post hoc* test: $P<0.05$). Values are presented as the mean ± SE, and asterisks indicate statistical significant differences.

Therefore ZAC protein is reduced in pituitary adenomas when compared to the normal pituitary gland, which is in agreement with the data derived by PCR and *in situ* hybridization at mRNA level. Our results demonstrate a dramatic decrease of the signal in non-functioning tumors, when compared to normal pituitary or clinically active tumors. When ZAC protein levels were compared with proliferation index or tumor invasiveness, no significant correlation was found, implying that loss of ZAC gene expression is an early event in pituitary tumorigenesis.

ZAC and methylation

ZAC gene promoter has a CpG island, suggesting that ZAC/Zac down-regulation can also be due to aberrant promoter hypermethylation. To test this hypothesis GH3 cells were treated with the anti-methylating agent 5'-aza-deoxycytidin (5-Aza-dC). The result was an up-regulation of Zac1 gene levels in a dose dependent manner, as shown in Fig.12, attributing the reduction of Zac1 expression in this cell line to promoter hypermethylation.

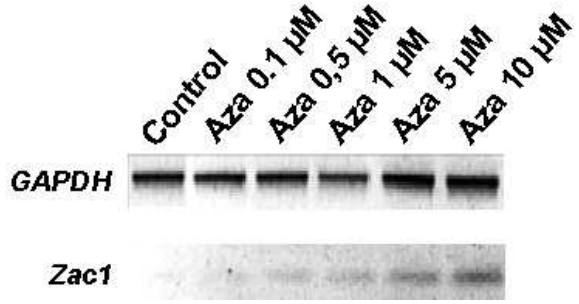

Fig.12. Effect of the demethylating agent 5'-aza-deoxycytidin on Zac1 gene expression in GH3 cells. Zac1 gene expression is increased in a concentration dependent manner after two days of stimulation with the agent. The picture is shown after inverting the original image, which was visualized by ethidium bromide staining.

Correlation between ZAC and EGFr expression in pituitary adenomas

In rat ovarian epithelial cells, EGF stimulation leads to ZAC downregulation, suggesting that probably the reduced ZAC levels observed in most non-functioning tumors can be due to high EGFr levels (Abdollahi et al., 1999). However, the controversial results about the status of EGFr in pituitary adenomas (indicated in the reviews of Dahia and Grossman, 1999; Ray and Melmed, 1997) cannot provide a definite picture about EGFr expression in these tumors. To elucidate this controversy, we initiated a new examination for the presence of EGFr mRNA and protein in a large number of pituitary tumors.

EGFr mRNA expression in normal and adenomatous human pituitary

To study the levels of EGFr mRNA in normal and adenomatous pituitary nested PCR was performed. This type of PCR was chosen because it gives information about the levels of the wild type form of EGFr (EGFrWT) and the presence of constitutively active EGFr variant III (EGFrvIII; Schlegel et al., 1994). This mutant EGFr derives from a 801 bp in-frame deletion in the extracellular part of the receptor and is found in some forms of cancer. As positive control for EGFrWT, a meningioma known to express high levels of EGFr was used. A plasmid having the fragment derived from the 800 bp deletion, was used as positive control for EGFrvIII.

RT/Nested PCR in all three normal pituitary glands resulted in the amplification of the predicted 1002 bp band corresponding to the wild type EGFr (EGFrWT). From the 47 adenomas examined, EGFrWT transcript was present in both Cushing's adenomas, 6/9 acromegaly associated tumors, 1/4 prolactinomas, and 19/30 non-functioning pituitary adenomas, while both thyrotrophinomas studied had no detectable EGFr mRNA (Fig.13). The constitutively active EGFrvIII was not detected in any tumors studied.

EGFr protein in normal human pituitary and pituitary adenomas

To assess the EGFr protein status, initially three mouse monoclonal antibodies were tested: two against the intracellular (clone F4 and clone c11/IgG1) and one against the extracellular (clone E30) part of the receptor. The clone c11/IgG1 was dropped because it produced unspecific staining in vascular structures and connective tissue.

A meningioma was included in every immunohistochemical reaction as positive control. In the normal pituitary gland, EGFr immunoreactivity was dispersed in 20-30% of the anterior pituitary cells (Fig.13 B), and the staining was abolished after preabsorption of the clone F4 antibody with its homologous antigen (Fig.13 C). Double-IHC revealed EGFr immunoreactivity in a small number of somatotrophs,

corticotrophs, lactotrophs, and gonadotrophs, which is in accordance with previous observations in rat anterior pituitary cells (Fan and Childs, 1995). In addition, EGFr was found to be expressed in a small number of folliculostellate cells, as revealed after colocalization with the folliculostellate cell marker S100. Immunohistochemistry on paraffin embedded pituitary tissues with the clone E30 confirmed these results.

To examine the expression of EGFr in the different types of pituitary tumors, immunohistochemistry was performed on 82 cases (17 acromegaly-associated adenomas, 11 prolactinomas, 7 Cushing's adenomas, 5 thyrotrophinomas, and 42 clinically non-functioning pituitary adenomas), all of which were cryostat sections and some also paraffin embedded. In this part of the study all the cases were taken collectively without making distinction other than the pituitary adenoma type to which they belong. Since the clone E30 antibody works only on paraffin embedded sections, comparison between the immunoreactivities derived from the two antibodies was made only in the cases that were both cryostat and paraffin embedded.

From the 40 clinically active pituitary adenomas involved in the study, 18 were immunopositive for EGFr, while only 6 out of 42 clinically inactive tumors displayed EGFr immunoreactivity. As shown in Table 3, the percentage of endocrine cells immunopositive for the receptor was 2-30% in acromegaly associated adenomas, prolactinomas, and thyrotrophinomas, reaching the 50% in most Cushing's adenomas, while in the 6 immunopositive NFPA, it did not exceed the 10%. It is therefore evident, that clinically active tumors express EGFr more frequently than non-functioning pituitary adenomas. It is of interest that the highest levels of expression occur in the Cushing's associated pituitary adenomas.

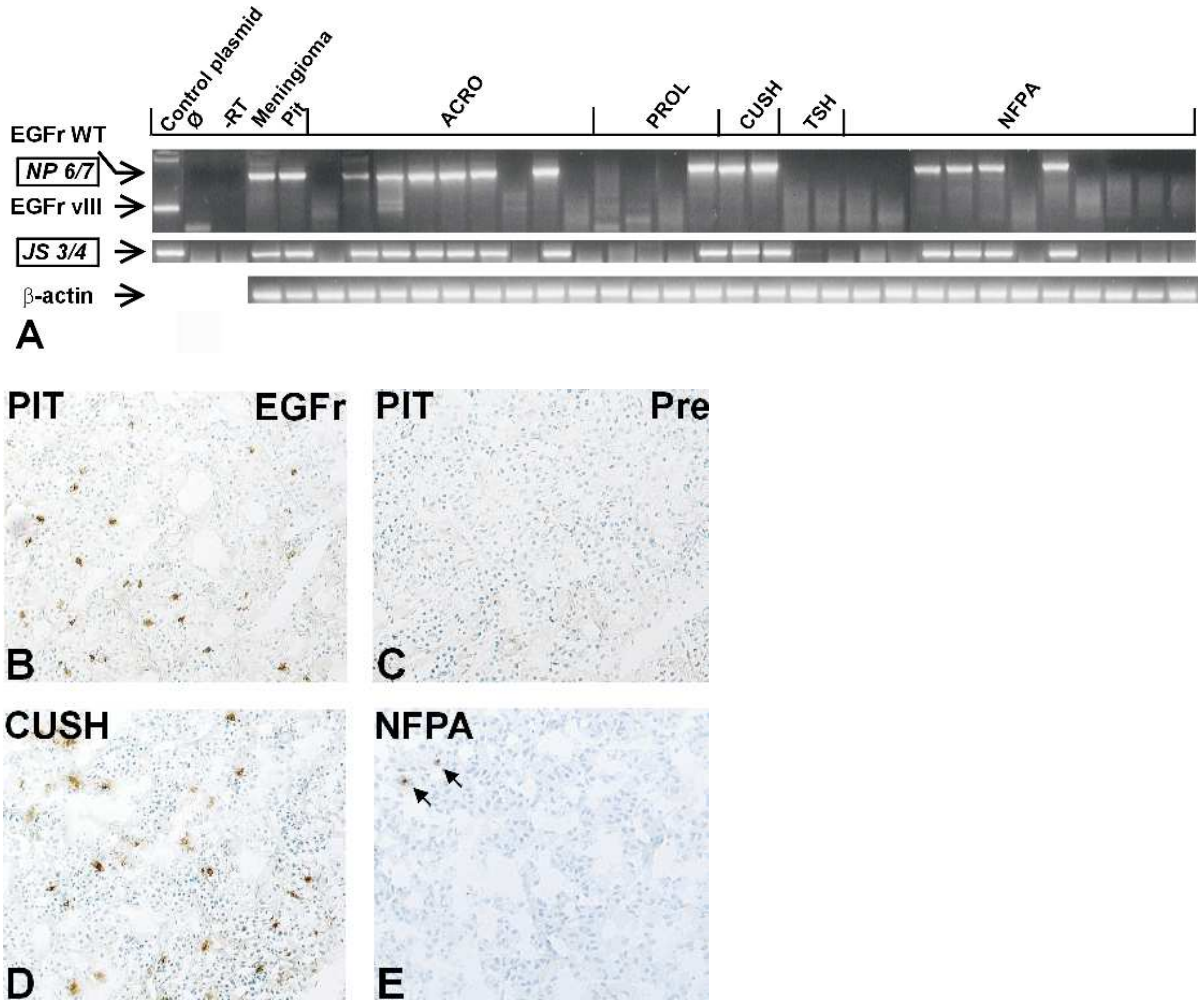

Fig.13. EGFr expression in normal and adenomatous pituitary. *A*. *Top panel*, nested PCR screening for EGFr WT (1002 bp) and EGFr vIII (201 bp) using the NP6/7 inner primer pair; Ø: PCR in absence of template; Control plasmid: shows the position of the band corresponding to the EGFr vIII; Meningioma: is used as positive control for the EGFr WT; Pit: normal anterior pituitary gland. *Second panel*, control PCR using the JS3/4 inner primer pair reveals presence of EGFr; Control plasmid for JS3/4 shows the position of the band corresponding to the EGFr fragment. *Third panel*, *β*-actin in the same set of tumors. *B*. EGFr protein (brown) in human adenohypophysis using the clone F4 antibody. EGFr immunopositive cells are scattered comprising around 30% of anterior pituitary cells. *C*. Parallel section incubated with the preabsorbed clone F4 antibody. Almost all the staining is abolished. *D*. EGFr staining in a representative Cushing's associated pituitary adenoma. Immunoreactive cells are abundant throughout the tissue. *E*. Representative EGFr immunopositive non-functioning pituitary adenoma. EGFr immunoreactivity is concentrated in a small number of cells (filled arrows) leaving most of the tissue immunonegative. All the four tissues are cryostat sections. Nuclei are counterstained with toluidine blue.

Tumor type	% of cases immunopositive for EGFR	Mean % of immunopositive cells
ACRO	28%	2-40%
PROL	42%	1-30%
CUSH	83%	10-50%
NFPA	28%	1-10%

Table 3. Distribution of EGFr immunopositivity among four pituitary tumor types: acromegaly-associated adenomas (*ACRO*), prolactinomas (*PROL*) Cushing's adenomas (*CUSH*), and non-functioning pituitary adenomas (*NFPA*). *Second column*: percentage of cases per tumor category which showed even the slightest EGFr immunoreactivity. *Third column*: mean percentage of EGFr immunopositive cells, determined as follows: three representative areas of the tumor were counted for cells expressing EGFr protein using a grid and the mean was calculated.

Correlation between ZAC and EGFr expression

To compare ZAC and EGFr expression in the same tumor specimens, 16 additional pituitary adenoma cases (5 acromegaly associated tumors, 2 prolactinomas, 1 thyrotrophinoma, 3 gonadotrophinomas, and 5 null cell adenomas) were examined by PCR for ZAC and by nested PCR for EGFr. Nested PCR revealed EGFr expression in 7 cases: 4 acromegaly-associated pituitary adenomas, 1 prolactinoma, 1 gonadotrophinoma, and 1 null cell adenoma. The immunohistochemical examination of these pituitary tumors, together with the 82 cases described above, revealed no correlation between ZAC absence and EGFr presence. In most NFPA, in which ZAC expression was reduced or lost, none or only few EGFr positive endocrine cells were present. In the few tumors showing strong EGFr immunoreactivity, double immunolabeling for EGFr and ZAC confirmed that ZAC expression was independent of EGFr status. An example is shown in Fig.14 in which double immunohistochemistry in a Cushing's adenoma, displaying low levels of ZAC and moderate number of EGFr immunopositive cells, revealed that some of these EGFr expressing cells could display ZAC immunoreactivity similar to the EGFr immunonegative cells.

Altogether, these results indicate that in the majority of non-functioning pituitary adenomas there is no correlation between loss of ZAC and presence of EGFr. In addition the finding of ZAC expression in EGFr positive cells suggest that EGFr may not play a role in the regulation of ZAC in pituitary tumors.

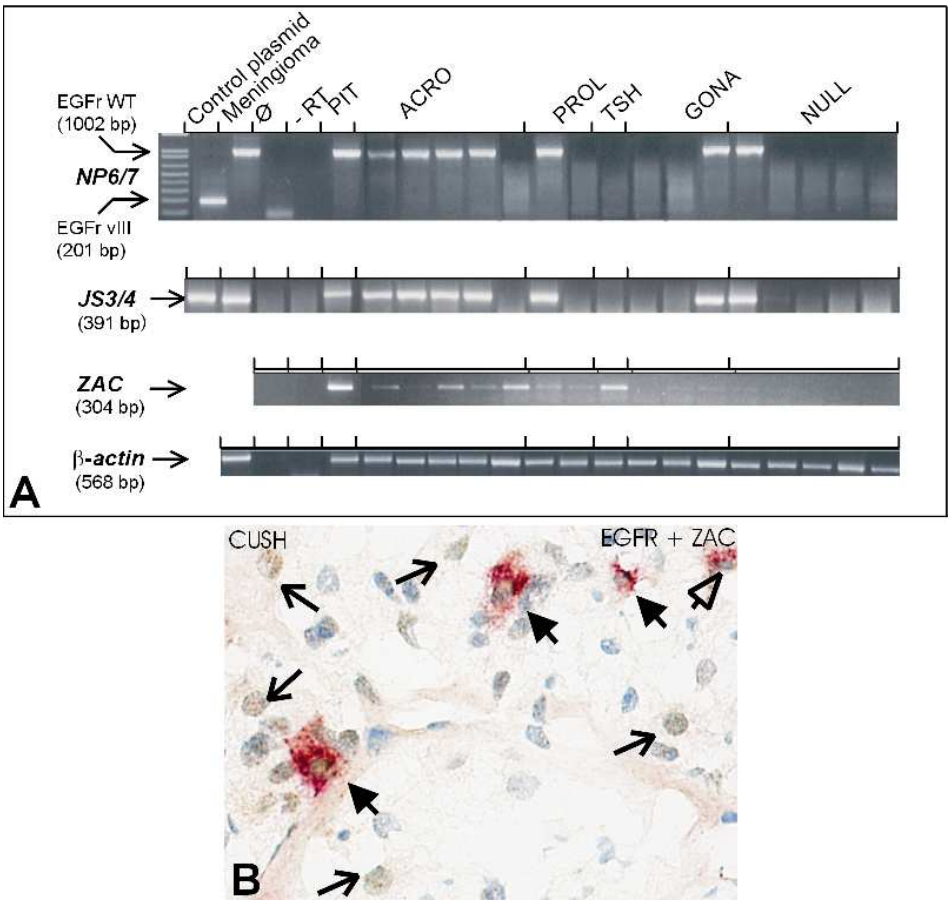

Fig.14. Comparison between ZAC and EGFr expression in 16 additional cases of pituitary tumors. *A. Top panel*, nested PCR screening for EGFr WT (1002 bp) and EGFr vIII (201 bp) using the NP6/7 inner primer pair; M: 1-kb Plus DNA Ladder (Life Technologies, Inc) ; Ø: PCR in absence of template; Control plasmid: shows the position of the band corresponding to the EGFr vIII; Meningioma: is used as positive control for the EGFr WT. *Second panel*, control PCR using the JS3/4 inner primer pair reveals presence of EGFr; Control plasmid for JS3/4 shows the position of the band (391 bp) corresponding to the EGFr fragment. *Third and fourth panel*, ZAC and *β*-actin expression in the same set of tumors. *B.* Double IHC for ZAC (brown) and EGFr (red) in a CUSH, revealing cells immunopositive for EGFr and negative for ZAC (*empty arrow*) and immunopositive for ZAC and negative for EGFr (*open arrows*), but also cells immunopositive (*filled arrows*) or immunonegative for both ZAC and EGFr (40x magnification). Nuclei are counterstained with toluidine blue.

Effect of EGF stimulation on ZAC gene expression

Our studies by PCR and immunohistochemistry showed no correlation between ZAC absence and EGFr expression. Moreover, they suggested that EGFr signaling may not influence ZAC gene expression in pituitary cells, as it is the case in ovarian epithelial cells. To prove whether this is the case or not, we studied the effect of EGF on Zac1 gene expression in the rat pituitary tumor cell line GH3. GH3 cells, despite the fact that they have the characteristics of mammosomatotroph cells, are characterized by very low basal levels of Zac1 gene expression (Fig.15 A), resembling, in this context, the non-functioning pituitary adenomas.

The stimulation with EGF led to reduction of Zac1 mRNA levels, and this effect was reversed by the MAPK inhibitor PD 098509 and the EGFr tyrosine kinase inhibitor tyrphostin AG 1478 (Fig.15 A). In addition, EGF stimulation in 27 cases of non-functioning pituitary adenomas in primary cell culture resulted in ZAC downregulation in 6 cases (Fig.15 C). All these 6 cases were proven to be EGFr immunopositive by PCR and immunohistochemistry.

Therefore, ZAC/Zac1 can be a target of a signaling pathway involving EGFr tyrosine kinase and MAPK activation (Fig.15 B). However, only in a small number of non-functioning pituitary adenomas which are expressing EGFr, can this mechanism be the reason for the reduction in ZAC gene expression.

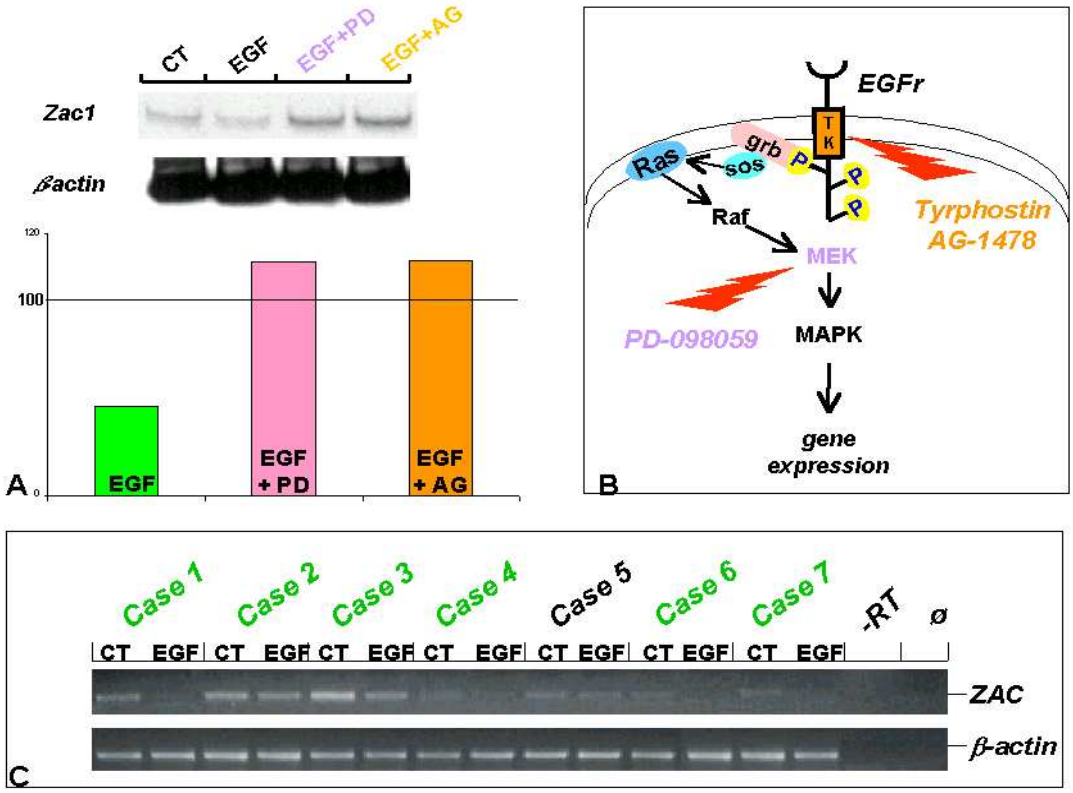

Fig.15. Effect of EGF on ZAC/Zac1 gene expression. *A.* Semiquantitative RT-PCR reveals a downregulation of Zac1 after stimulating GH3 cells with 1μM EGF. This inhibitory effect is reversed when co-stimulating with 1μM PD-098059 (EGF+PD) or 1μM tyrphostin (EGF+AG). In the graph are shown the values of each stimulation, presented as a percentage of the unstimulated control. *B.* Schematic presentation of the major components of the EGFr signaling pathway. With red arrows are indicated the steps of the pathway inhibited by the PD-098059 and tyrphostin (AG-1478). *C.* RT-PCR on the 6 cases of non-functioning pituitary adenomas in which EGF resulted in ZAC downregulation. Case 5 is a representative of the majority of non-functioning pituitary tumors, in which EGF has no effect.

Effect of octreotide on Zac1 gene expression in GH3 cells

The next step in this study was to examine whether ZAC/Zac1 gene expression can be regulated by compounds, frequently used for the treatment of pituitary adenomas. The GH3 cell line is not an appropriate model to study the effect of dopamine agonists in Zac1 gene expression because it does not express D2R (Dr M. Paez-Pereda, personal communication); therefore this study concentrated on the effect of the somatostatin analogue octreotide.

Octreotide treatment in GH3 cells resulted in decreased proliferation, but not in apoptosis, as determined by the acridine orange/ethidium bromide staining. The antiproliferative effect of octreotide was reversed when pretreating with pertussis toxin, indicating the involvement of Gi protein (Fig.16 A). This antiproliferative effect was accompanied by a significant increase in Zac1 levels (Fig.16 B). The stimulatory effect of octreotide on Zac1 was reversed after pretreatment with pertussis toxin, and after co-stimulation with the phosphatidylinositol 3-Kinase (PI3K) inhibitor, LY 294002 (Fig.16 C).

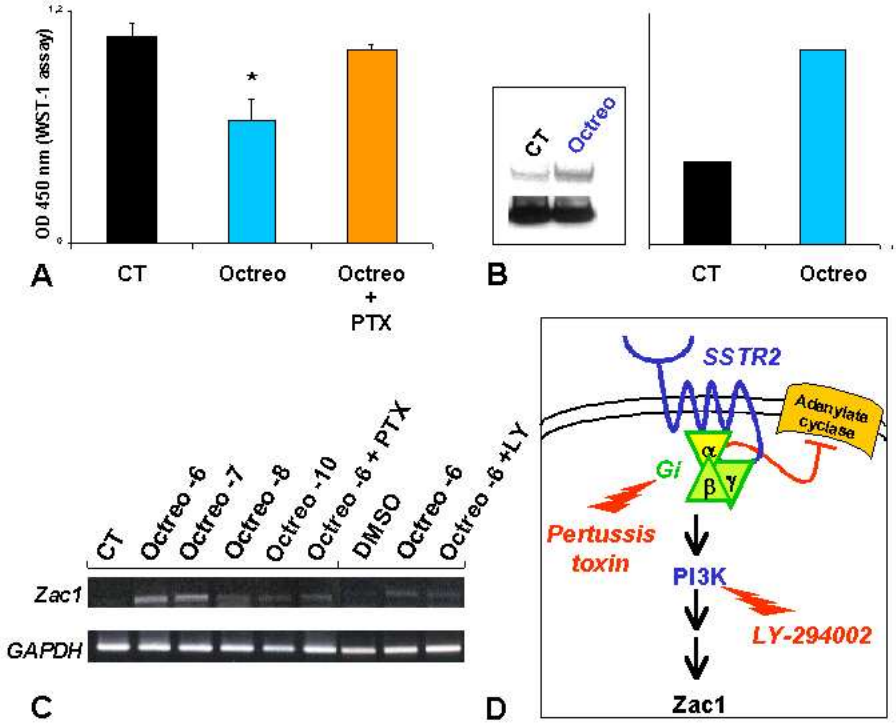

Fig.16. **Effect of Octreotide on ZAC/Zac1 gene expression.** *A.* Results of WST1 assay demonstrating the inhibitory effect of 1μM octreotide (Octreo) on GH3 cell proliferation (One-Way ANOVA: $P<0.05$). This effect reversed after 6 hours pretreatment with 100 ng/μl pertussis toxin (PTX). *B.* Increased Zac1 gene expression after stimulating for 24 hours with 1μM octreotide, as determined by semiquantitative RT-PCR. *C.* RT-PCR showing Zac1 expression after stimulating with octreotide with concentrations 1μM (Octreo-6), 100nM (octreo-7), 10nM (Octreo-8), and 0.1nM (Octreo-10). 1μM octreotide failed to upregulate Zac1 when applied in cells pretreated for 6 hours with 100ng/μl pertussis toxin (Octreo-6+PTX) or co-stimulated with 1μM LY-294002 (Octreo-6+LY). *D.* Schematic presentation of the major components of the SSTR2 signaling pathway.

Discussion

In this thesis, it is demonstrated that ZAC mRNA and protein are highly expressed in human adenohypophysis but its levels are reduced in most pituitary adenomas and dramatically reduced or absent in non-functioning pituitary adenomas. These findings are of high interest in light of the antiproliferative properties of ZAC (Pagotto et al., 1999). Additionally, ZAC is located to chromosome 6q24-q25, a region known to harbor putative tumor suppressor genes involved in solid tumor development (Foulkes et al., 1993). Eight out of 18 samples displayed LOH for at least one informative marker, but no mutations were found in the ZAC coding region in these tumors (Pagotto et al., 2000). However, as shown in this thesis, ZAC mRNA and protein were lost or reduced in non-functioning adenomas with statistical significance. An interesting finding was the greater decrease of ZAC mRNA and protein expression in null cell adenomas in comparison to gonadotrophinomas. Null cell adenomas are tumors of gonadotroph origin displaying little or no immunoreactivity to gonadotrophins and are thought to be de-differentiated terminal tumoral entities (Holm, 1995). Therefore, the loss of ZAC in the less differentiated null cell adenomas suggest that ZAC may play a role in pituitary development and more specifically in the differentiation of the gonadotroph lineage. Furthermore, our data provide more evidence for ZAC to be considered as a TSG.

No correlation was found between ZAC expression and proliferation index or invasiveness of pituitary adenomas, indicating that loss of ZAC expression may be an early event in pituitary transformation. One early molecular process that might act as an alternative mechanism of gene silencing is gene methylation. Recent studies have shown that ZAC is an imprinted gene, and that its promoter is having at least one CpG island, making ZAC gene expression susceptible to hypermethylation (Varrault et al., 2001). Treating different human breast cancer cell lines with the de-methylating agent 5-Aza-dC resulted in ZAC re-expression (Varrault et al., 1998). In

the pituitary tumor model GH3, treatment with 5-Aza-dC resulted in dose dependent increase in Zac1 expression, suggesting the possibility of gene silencing due to promoter hypermethylation, also in the case of pituitary adenomas. However, the limitations, set by the low proliferation rate of primary pituitary tumors in culture, prevented similar experiments in non-functioning tumors.

Aberrant signaling by growth factors and hypothalamic peptides in an auto- or paracrine manner may contribute to the transformation processes taking place during pituitary tumorigenesis. EGF was shown to downregulate Zac1 gene expression in rat ovarian epithelial cells, suggesting that aberrant EGFr signaling pathway can be responsible for the downregulation of ZAC expression in pituitary tumors. However, the previous controversial results about the status of EGFr in pituitary adenomas have made it difficult to establish a role for EGF in pituitary oncogenesis (Dahia and Grossman, 1999; Ray and Melmed, 1997)).

The present study attempted to solve this controversy by examining the presence of the receptor at mRNA and protein level. All the techniques pointed out to the fact that EGFr is more frequently expressed in clinically active than in non-functioning pituitary adenomas. These data are in contrast with a previous study that has shown EGFr ir only in clinically inactive adenomas but not in the hormone secreting tumors (Chaidarun et al., 1994). However, they are partially in agreement with another study that had shown EGFr in half clinically active adenomas and in most non-functioning adenomas (LeRiche et al., 1996).

The expression of ZAC and EGFr was assessed in a number of pituitary adenomas together with the presence of EGFvIII, a constitutively active variant of EGFr which is highly expressed in malignant tumors. No correlation was observed between ZAC and EGFr status, and most non-functioning adenoma cases studied were completely devoid of EGFr expression.

The data derived from our studies, forced us to speculate that regulation of ZAC gene expression in pituitary tumors is not susceptible to the EGFr signaling pathway. However, stimulation of the pituitary mammosomatotroph cell line GH3 with EGF led to further decrease in Zac1 mRNA levels, in signaling pathway involving EGFr tyrosine kinase and MAPK activation. In addition, in the few cases of non-functioning pituitary adenomas expressing EGFr, EGF downregulated ZAC gene expression. These data lead to the conclusion that ZAC/Zac1 gene expression can be under the control of the EGFr signaling pathway in pituitary cells. However, due to the absence of EGFr expression in non-functioning pituitary adenomas, factors other than EGF must be responsible for the low levels of ZAC in these tumors.

Having in mind the antiproliferative role of ZAC/Zac1, it is of great importance to find a way to pharmacologically manipulate its expression. In the present study, the somatostatin analogue octreotide was investigated in respect to Zac1 gene expression using the GH3 cell line as a model. Octreotide acts through the somatostatin receptor 2 (SSTR2), which is coupled to the Gi protein (Gu and Schonbrunn, 1997) therefore inhibiting adenylate cyclase and cAMP production (Chen et al., 1997), and to PLC and inositol lipid pathway (Tomura et al., 1994). Despite the fact that octreotide is known to inhibit growth in several cell models, its mode of action is not fully elucidated. It has been demonstrated that SSTR2 mediate G1 cell cycle arrest by increasing p27/Kip1 levels, through a pathway involving the tyrosine phosphatase src homology 2-containing tyrosine phosphatase 1 and inhibition of MAPK (Pages et al., 1999).

In this thesis, it is shown that octreotide inhibits GH3 cell proliferation and increases Zac1 mRNA levels. Both effects were mediated by a pertussis toxin sensitive pathway. The stimulatory effect of octreotide on Zac1 gene expression was reversed after co-stimulating with the PI3K inhibitor LY-294002, implicating the PI3K signaling in the regulation of Zac1 gene expression.

The antiproliferative action of octreotide on GH3 cells has been already demonstrated in a previous study, which revealed that octreotide blocks the G0/G1 cell cycle transition (Cheung and Boyages, 1995). It is of interest that ZAC/Zac1 induces cell cycle arrest acting at this cell cycle point (Spengler et al., 1997). A recent study, comparing proliferation index and apoptosis of a series of octreotide treated and untreated acromegalic tumors, showed that the proliferation index is much lower in treated than in untreated tumors, while there is no difference in the apoptotic index (Losa et al., 2001). This is in agreement with our observations in GH3 cells, in which treatment with octreotide resulted in a decrease in proliferation but not in apoptosis. Moreover, in our study the highest levels of ZAC expression were observed in acromegaly associated tumors, all of which were treated with somatostatin analogues before the neurosurgical operation. This observation adds to the speculation of a stimulatory effect of somatostatin analogues on ZAC gene expression. These findings make Zac1 a candidate mediator of the antiproliferative effects of octreotide.

Altogether these data implicate the novel putative tumor suppressor gene ZAC in the pathogenesis of non-functioning pituitary adenomas. Although downregulation of the gene can be attributed to the EGFr signaling in the few cases, expressing the receptor, the mechanism responsible for ZAC reduction or loss in the majority of non-functioning tumors remains obscure. Our preliminary results refer to gene silencing by promoter hypermethylation as an interesting mechanism that may apply in this type of tumors, although additional studies must be performed to prove this speculation. On the other hand, the strong effect of octreotide, in upregulating ZAC gene expression, provides a novel mechanism for the antiproliferative action for this drug and suggest to reconsider the option of using somatostatin analogues for the pharmacological treatment of non-functioning adenomas. Future studies will aim to elucidate the status of SSTR expression and function in this type of pituitary

adenomas, as well as the efficiency of treatment with novel, more specific and potent somatostatin analogues. The elucidation of the mechanisms responsible for the regulation of ZAC gene expression, is of high importance since it may pave the way for the development of new therapeutical approaches, more applicable to this type of pituitary tumors whose growth cannot be pharmacologically limited at present.

RESULTS ON MENIN

MEN1 mRNA in normal and adenomatous pituitary

MEN1 gene expression was assessed using RT-PCR in 2 normal anterior pituitary glands, and 20 pituitary adenomas. The MEN1 transcript was amplified in all 20 adenomas, which is consistent with previous reports (Asa et al., 1998; Prezant et al., 1998; Farrell et al., 1999) that have demonstrated intact MEN1 gene expression in all sporadic pituitary adenoma cases.

Menin expression in normal human pituitary

Using a monoclonal antibody against the menin C-terminus (mAb#4-15; Ferro, et al.), immunohistochemical examination was performed in 7 human pituitary glands. Menin immunoreactivity was present in almost all endocrine cells of the anterior lobe, and in some pituicytes of the posterior lobe (Fig.17 A). The staining was nuclear, which is in agreement with previous observations in transfected cells (Guru et al., 1998). The specificity of the signal was determined by using the mouse pre-immune serum (Fig.17 B) and after preabsorbing the mAb #4-15 antibody with the immunogen (Fig.17 C).

Double immunohistochemistry revealed menin immunoreactivity in all types of hormone producing cells and in folliculostellate cells, indicating that its expression is not restricted to one particular cell population (examples in Fig.17 E and F). The nuclei of the fibroblasts and endothelial cells were devoid of any menin immunoreactivity.

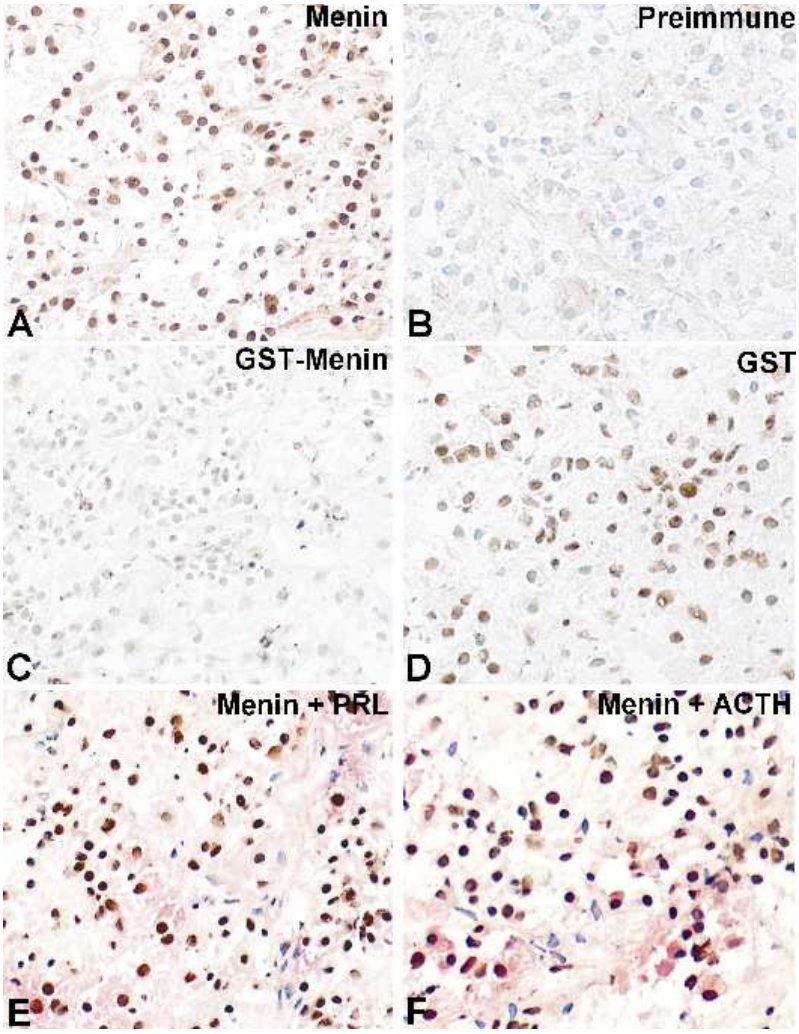

Fig.17. Menin expression in human adenohypophysis. *A*. Menin is present in the nuclei of all endocrine cells but not in the nuclei of endothelial cells. The specificity of the mAb #4-15 is demonstrated by depletion of the signal after incubation with the preimmune serum (*B*) or after preabsorption of the antibody with the GST-Menin immunogen (*C*). Preabsorption with the GST alone doesn't alter the antibody activity (D). Menin colocalizes with all the adenohypophyseal hormones; e.g. with PRL (*E*) and ACTH (*F*).

Menin expression in pituitary tumors

Immunohistochemical analysis of 58 sporadic pituitary adenomas (listed in Table 4) revealed the same nuclear distribution of menin staining as in the normal adenohypophyseal cells. Although all the endocrine cells in each tumor were expressing menin, there was a big fluctuation in the intensity of the immunoreactivity, which varied from levels comparable to the normal pituitary (Fig.18 A) down to weak, almost undetectable signal (Fig.18 C). In brief, only 21% of the adenomas examined

had strong, i.e. comparable to that of the normal adenohypophysis, signal for menin. Another 33% displayed an average level of expression, and the remaining 47% demonstrated weak menin immunoreactivity. The intensity correlates neither with the histological and clinical features of the tumor nor with the grade and invasiveness (Table 4).

A rare case of a PRL-secreting pituitary carcinoma was included in the study. This adenoma had been operated twice before it finally transformed into carcinoma (Winkelmann et al, 2001). The samples derived from the first and the second operations still retained weak menin immunoreactivity (Fig.18 D), while the autoptical tissues obtained three years later from a parasellar and orbital invasion and from metastases derived from medulla oblongata, spinal canal and left femur, were menin immunonegative (example medulla oblongata in Fig.18 E).

A pituitary adenoma derived from a patient with familiar MEN1 syndrome was also included in the study. In this tumor, the MEN1 gene in one allele was lost and in the other carried a mutation (Cetani et al., 1999). Immunohistochemistry for menin resulted in no signal (Fig.18 G), confirming the specificity of the mab#4-15 antibody.

The possibility of poor preservation was excluded by immunohistochemistry for the nuclear proteins Pit1 in the case of acromegaly-associated tumors and prolactinomas, and SF1, for non-functioning pituitary adenomas.

		Age/Sex	Diagnosis	IHC	G	Menin ir
1.	460	28/M	ACRO	GH	III	++
2.	511	43/M	ACRO	GH/PRL	III	+
3.	521	44/F	ACRO	GH	II	+++
4.	559	35/F	ACRO	GH	III	++
5.	384	36/M	ACRO	GH/PRL	II	+
6.	506	33/M	ACRO	GH	III	+
7.	508	31/F	ACRO	GH	III	++
8.	549	64/F	ACRO	GH/α-sub/FSH	III	+
9.	572	65/F	ACRO	GH	III	+
10.	513	50/M	ACRO	GH/PRL	III	+
11.	575	64/M	ACRO	GH/PRL	III	+++
12.	699	36/F	ACRO	GH/PRL	III	++
13.	314	64/M	ACRO	GH/PRL	II	+++
14.	445	80/M	ACRO	GH/PRL	II	+
15.	545	42/F	ACRO	GH/PRL/α-sub	III	+++
16.	553	32/M	ACRO	GH/PRL	II	++
17.	624	58/F	ACRO	GH/PRL	II	+++
18.	555	52/F	ACRO	GH/FSH/LH	II	+
19.	455	30/M	CUSH	ACTH	I	++
20.	473	16/M	CUSH	ACTH	I	+++
21.	482	24/F	CUSH	ACTH	II	++
22.	528	62/F	CUSH	ACTH	II	+
23.	529	34/M	CUSH	ACTH	I	+++
24.	563	24/F	CUSH	ACTH	II	+
25.	306	*44/F*	PROL	PRL	II	++
26.	310	63/M	PROL	PRL	III	++
27.	488	42/F	PROL	PRL	III	+
28.	495	48/F	PROL	PRL	III	+
29.	523	37/F	NFPA	PRL	III	+
30.	591	43/F	PROL	PRL	III	++
31.	570	42/M	NFPA	ACTH	III	++
32.	597	69/M	NFPA	ACTH	II	+
33.	383	51/F	NFPA	α-sub	II	++
34.	312	63/M	NFPA	LH/α-sub	II	++
35.	391	51/F	NFPA	FSH /LH	II	++
36.	403	72/M	NFPA	FSH/LH	II	+++
37.	464	61/M	NFPA	FSH	II	+++
38.	538	46/M	NFPA	FSH	III	+
39.	539	28/M	NFPA	FSH	III	+
40.	550	50/M	NFPA	FSH	III	++
41.	551	35/M	NFPA	FSH	III	+
42.	566	76/M	NFPA	α-sub/FSH/LH	II	+++
43.	582	31/F	NFPA	FSH	II	+
44.	587	37/F	NFPA	α-sub/FSH/LH	III	++
45.	629	65/M	NFPA	FSH	III	+
46.	698	46/F	NFPA	LH	II	+
47.	700	53/M	NFPA	FSH/LH/α-sub	II	++
48.	406	48/M	NFPA	None	III	++
49.	407	*77/M*	NFPA	None	II	+++
50.	446	70/M	NFPA	None	III	+
51.	481	63/F	NFPA	None	II	+
52.	574	60/M	NFPA	None	II	++
53.	577	52/F	NFPA	None	III	+
54.	585	49/M	NFPA	None	III	+
55.	609	59/F	NFPA	None	III	+
56.	627	69/F	NFPA	None	II	+
57.	498	51/F	NFPA	None	III	+
58.	509	73/F	NFPA	None	II	+++
59.	389	50/M	PROL	PRL	IV	+
60.	397	50/M	PROL	PRL	IV	+
61.	Para	53/M	PROL	PRL	IV	0
62.	Orbi	53/M	PROL	PRL	IV	0
63.	Med	53/M	PROL	PRL	IV	0
64.	Fem	53/M	PROL	PRL	IV	0

Table 4. List of the 58 tumors used for the menin study plus the tissues derived from pituitary carcinoma [# 59: 1st intervent; # 60 2nd intervent; # 61 parasellar invasion; # 62 orbital invasion; # 63 metastasis to medulla oblongata; # 64 metastasis to femural bone. The samples # 61-64 were taken at autopsy]. Information is given about the age, sex, clinical diagnosis of each patient. Tumor grade is given in column [G]. In the column [IHC] are listed the finding of the immunohistochemical examination for the 5 hormone and α-subunit in each case. Menin immunoreactivity (Menin ir) was determined by two independent investigators and categorized in 4 classes: (0): no Menin ir; (+) weak ir; (++) moderate ir; (+++) strong ir.

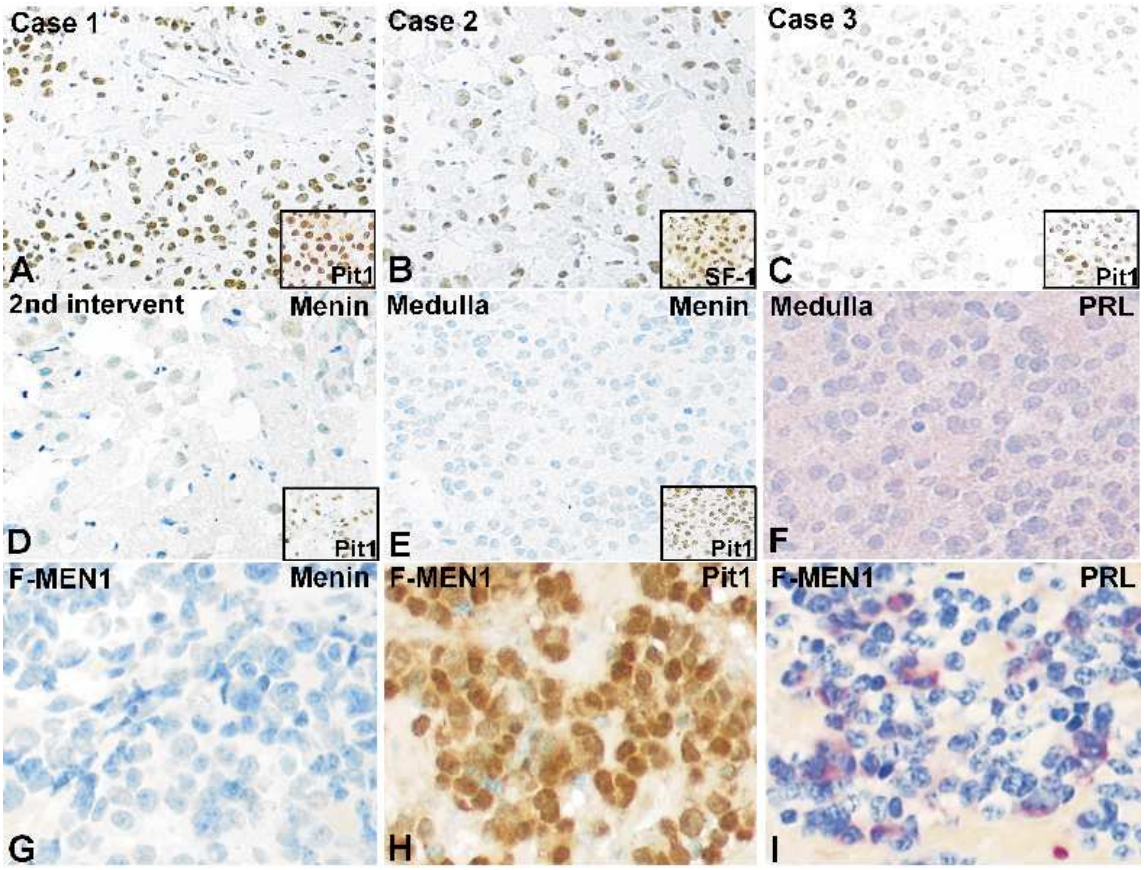

Fig.18. Menin immunoreactivity in pituitary adenomas. *Upper row*. Representative picture from each type of menin ir in sporadic pituitary adenomas. Menin ir varied from *A.* strong to *B.* moderate; and *C.* weak. *Middle row*. Menin ir in tissues derived from different stages of a PRL-producing carcinoma progression. *D.* Menin ir is weak in tumor obtained at the second intervent, and *E*. is totally absent in the autoptic tissues from the metastasis to the medulla oblongata. *F.* Immunostaining for PRL of a parallel section proves the pituitary origin of the tissue. The insets in pictures A to E show immunostaining for the transcription factors Pit1 or SF-1. *Last row*. Menin ir in a PRL-producing tumor

from a patient with familiar MEN1 syndrome. *G*. No menin ir is detected in the tumors specimen. *H*. The suitability of the tissue is proven by a strong ir for the transcription factor Pit1. *I*. Moderate PRL immunoreactivity in a section parallel to that displayed in [G].

Discussion

The fact that one of the most common manifestations of the polyendocrine syndrome MEN1 is the development of pituitary adenomas together with the increasing evidence that *MEN1* is a putative tumor suppressor gene, has drawn the attention to a possible role of this gene in the pathogenesis of sporadic pituitary adenomas. Immunohistochemistry in human normal pituitary gland revealed that most endocrine cells of the adenohypophysis displayed high levels of menin expression. Analyzing a large number of pituitary adenomas, it became evident that, despite the fact that most of the endocrine cells composing the adenoma were displaying a nuclear staining for menin, the highest percentage of the cases had weaker signal when compared to the normal adenohypophysis. There was no correlation between menin immunoreactivity and clinical and immunohistochemical diagnosis of each tumor studied, which is consistent with the observation that pituitary adenomas in MEN1 patients are not restricted to a certain clinical or histological type. In addition, there was no correlation between menin intensity and tumor grade and invasiveness. However, it is of interest that in the sole case of pituitary carcinoma included in our study, there was a complete loss of menin immunoreactivity in the latest specimens of the tumor, despite the retention of signal in the earlier specimens, indicating a possible role of menin in the transition from a benign phenotype to malignancy.

One possible explanation for the weak menin immunoreactivity could be loss of heterozygosity at the *MEN1* locus. Menin is biallelically expressed, therefore loss of one allele would theoretically result in decreased protein levels. LOH in 11q13, the MEN1 gene locus, was described in 5-20% of sporadic pituitary adenomas (Boggild et al., 1994). In the series of pituitary adenomas used in this study, LOH was

detected in 4 out of 12 cases (Theodoropoulou et al). All the 4 cases (# 6, 9, 38, and 39 in Table 4) were scored weak for menin. On the other hand, two cases of the remaining 8 specimens (# 2, and 43), which did not have LOH, also had weak menin immunoreactivity.

Analysis of the gene transcription by RT-PCR revealed no variation in the *MEN1* mRNA levels between neoplastic and non-tumorous pituitaries, confirming previous studies (Asa et al., 1998; Farrell et al., 1999; Satta et al., 1999). Therefore alterations in the transcriptional regulation of the gene do not account for the reduction of menin documented at protein levels by immunohistochemistry, which brings the suggestion that defects in the translational and postranslational processing may be responsible for the reduced levels of menin in sporadic pituitary adenomas.

This hypothesis is not paradoxical, since there are cases of proteins involved in cell cycle and tumor formation, which are regulated at protein level and their fate is determined by postranslational modifications. One paradigm is p27/Kip1, a regulator of cell cycle progression, which despite normal expression, its protein levels are reduced in a significant percentage of pituitary adenomas (Lidhar et al., 1999; Jin et al., 1997)

Although the exact mechanism is not yet clarified for the case of pituitary adenomas, abnormalities in the ubiquitin-mediated degradation system have been shown to lie behind the downregulation of p27/Kip1 in other types of tumors (Pagano et al., 1995; Loda et al., 1997). In conclusion, this study provides, for the first time, information about the pattern of menin expression in the normal and adenomatous pituitary. In human pituitary, menin is highly expressed in the anterior lobe, while tumorous transformation is associated with reduction in menin levels in a significant percentage of pituitary adenomas. Menin is a candidate tumor suppressor gene, which is speculated to play an important role in cell cycle regulation; therefore its decrease in sporadic pituitary adenomas may be a factor contributing in pituitary tumor formation.

Future studies will address whether defects in the translational or postranslational machinery are responsible for the abnormal regulation of menin levels.

RESULTS ON COUP-TFI

COUP-TFI mRNA in normal and adenomatous pituitary

RT-PCR for COUP-TFI was performed in two normal pituitaries and six corticotrophinomas. Both normal pituitaries expressed COUP-TFI mRNA, while all the six tumors failed to amplify the 440 bp product corresponding to the COUP-TFI, demonstrating that this factor is not expressed in Cushing's associated adenomas.

COUP-TFI protein expression in normal pituitary

To examine if COUP-TFI is physiologically expressed in the corticotrophs of the human normal adenohypophysis, its protein expression was assessed by immunohistochemistry. COUP-TFI protein was found to be present in 10-20% endocrine cells of the adenohypophysis (Fig.19 A). Double immunohistochemistry revealed COUP-TFI immunoreactivity almost exclusively in ACTH immunopositive cells (Fig.19 B). However COUP-TFI was found in no more than 20% of corticotroph cells. It is of interest that the highest percentage of COUP immunopositive cells was concentrated in the corticotroph-rich area found proximal to the rudimentary intermediate lobe of the hypophysis (Fig.19 C).

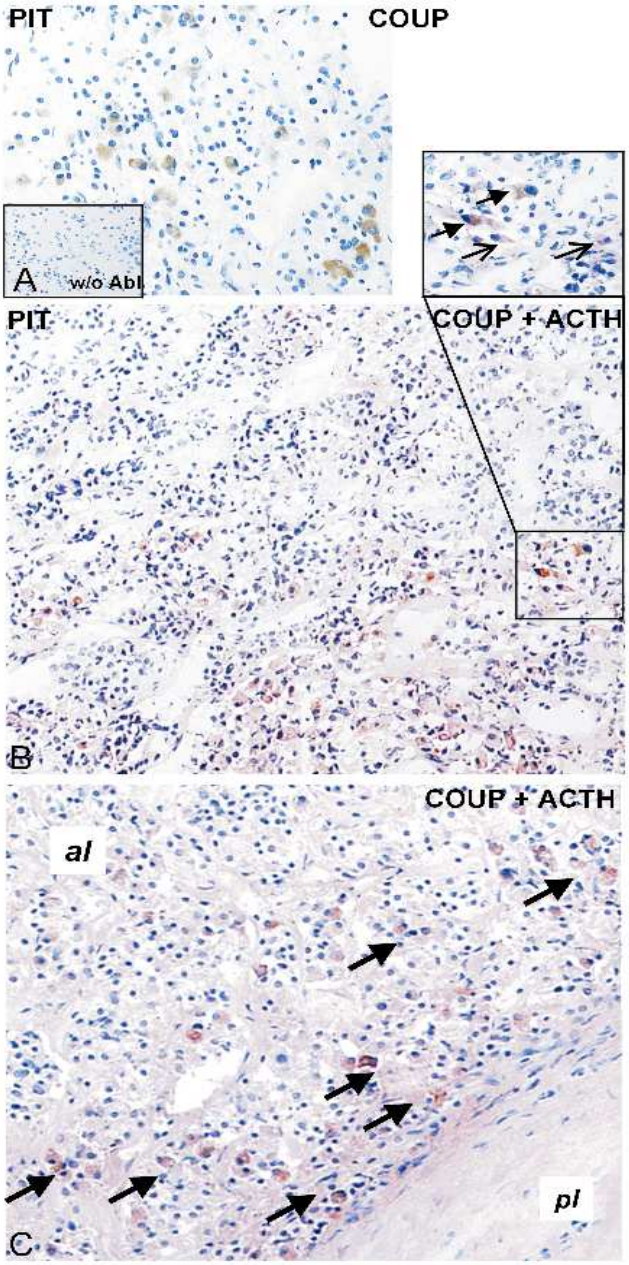

Fig.19. Immunohistochemical localization of COUP-TFI in human normal adenohypophysis. *A*. COUP-TFI immunopositive cells (*brown*) in the human normal anterior pituitary (10x magnification). In the inset is shown a parallel section in absence of the primary antibody (*w/o Abl*). *B*. Double immunohistochemistry with ACTH (*red*) and COUP-TFI in human adenohypophysis reveals a small number of ACTH-cells immunopositive for COUP (*dark red*; 5x magnification). In the inset above, a region containing cells immunoreactive for both ACTH and COUP (*filled arrows*) or only for ACTH (*open arrows*) is shown at higher magnification (20x). *C*. Double immunohistochemistry with ACTH and COUP-TFI, revealing the abundance of COUP-TFI immunopositive ACTH-cells in the border of the anterior lobe (*al*) and posterior lobe (*pl*) of the pituitary gland.

COUP-TFI protein expression in Cushing's and silent corticotroph adenomas

Immunohistochemistry was performed in seven Cushing's associated pituitary adenomas, all of which were completely immunonegative for COUP-TFI (Fig.20 A). In addition, 13 cases of silent corticotrophinomas were analyzed and all but one displayed no COUP-TFI immunoreactivity (Fig.20 B). The one silent corticotrophinoma, immunopositive for COUP-TFI, had parts with a moderate number of ACTH immunopositive cells and a big area with no ACTH immunoreactivity. It is of interest that the areas immunopositive for ACTH and COUP overlapped (Fig.20 C and D), and that the piece immunonegative for ACTH was also totally immunonegative for COUP (Fig.20 E and F).

These data confirm the observation at mRNA level, that COUP-TFI is not expressed in the corticotrophs of the Cushing's adenomas and of the silent corticotrophinomas.

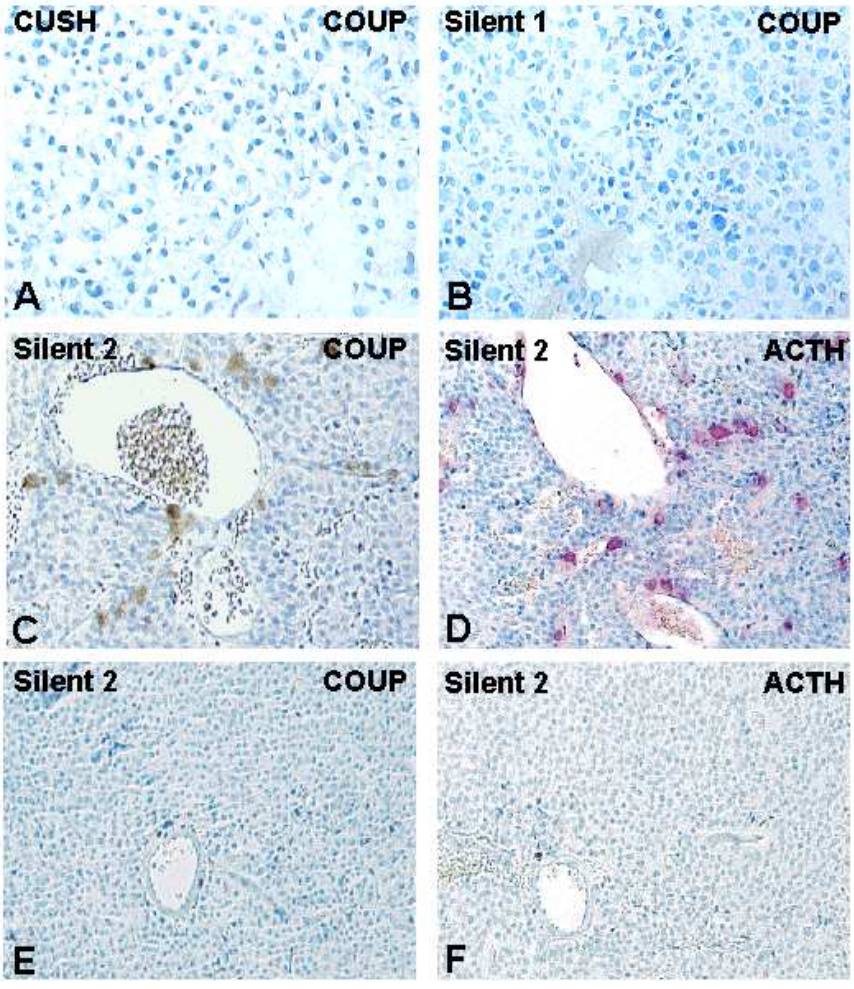

Fig.20. COUP-TFI expression in corticotrophinomas. *A.* Complete absence of COUP-TFI immunoreactivity in a representative Cushing's associated adenoma and *B.* a silent corticotrophinoma *C.* COUP immunoreactivity in the only immunopositive silent corticotrophinoma and *D.* ACTH staining in a parallel section. Notice the similarity in the COUP and ACTH staining patterns. *E* and *F*. A representative area of the same silent corticotrophinoma, demonstrating total absence not only of immunoreactivity for COUP-TFI but also for ACTH.

Effect of COUP-TFI overexpression on retinoic acid modulated POMC promoter activity

This investigation originated from the search for a factor that can be responsible for the absence of retinoic acid effect on ACTH production from the normal pituitary cells, despite the inhibitory effect in corticotrophinoma cells. COUP-TFI was shown to be expressed in normal but not in tumoral corticotrophs and is therefore a candidate factor responsible for this controversial effect.

To prove the effect of COUP-TFI on the POMC promoter, the factor was overexpressed in the mouse corticotrophinoma cell line AtT-20. Cells overexpressing COUP-TFI were transfected with plasmid containing luciferase gene downstream to the POMC promoter, and stimulated with retinoic acid. The effect of retinoic acid on POMC promoter was determined by detecting the luciferase activity. AtT-20 cells expressing a vector which was not containing the COUP-TFI gene (empty vector) were used as negative control.

In cells transfected with the empty vector, retinoic acid stimulation resulted in significant decrease in the POMC promoter activity, while in cells overexpressing COUP-TFI, it had no effect, clearly indicating that COUP-TFI acts as a transcriptional repressor for the retinoic acid effect on POMC promoter.

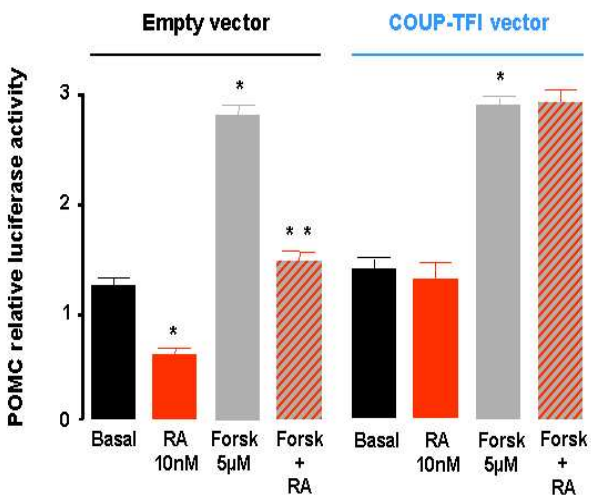

Fig.21. In AtT-20 cells transfected with 400ng vector that doesn't express the COUP-TFI gene (*empty vector*) stimulation with 10nM retinoic acid (*RA*) resulted in decrease of basal and forskolin (Forsk) induced POMC relative luciferase activity. This effect was not observed in the cells transfected with the COUP-TFI containing vector (*COUP-TFI vector*).

Discussion

It was recently shown that retinoic acid can revert Cushing's syndrome in an experimental animal model and inhibit ACTH synthesis from mouse and human corticotrophinomas but not from normal pituitary cells (Paez-Pereda et al., 2001). To elucidate the molecular basis of this difference in retinoic acid response, attention was focused to the transcription factor COUP-TFI, which is a well known retinoic acid signaling inhibitor (Kliewer et al., 1992; Tran et al., 1992; Cooney et al., 1993).

Our RT-PCR data reveal COUP-TFI expression in the normal but not in the adenomatous pituitary. Investigation of the COUP-TFI protein expression pattern by double immunohistochemistry, demonstrated the exclusive expression of this factor in corticotroph cells of the human adenohypophysis. Therefore the differential expression of this retinoic acid signaling inhibitor may be responsible for the different response of normal and tumoral corticotrophs to retinoic acid treatment. In addition, transfection studies in AtT-20 cells, showed that COUP-TFI overexpression reverts retinoic acid induced inhibition of POMC promoter activity. On the other hand, transfection with COUP-TFI had no noticeable effect on the basal POMC promoter activity, suggesting that probably COUP-TFI itself is not of high importance for the proper POMC gene transcription in AtT-20 cells.

The finding of abundant COUP-TFI expression in the corticotroph rich area of the intermediate lobe is of certain interest. Despite the fact that in rodents the intermediate lobe (or *pars intermedia*) is an organ with a certain physiological function, in humans its significance is under debate. However, there is evidence that the *pars intermedia* is the source of the clinically silent corticotroph adenoma, a rather enigmatic type of pituitary adenoma, which although stains immunopositive for ACTH, it is not associated with alterations in cortisol levels and Cushing's syndrome (Horvath et al., 1980; Lamberts et al., 1982; Scheithauer et al., 2000). In our study, the examination of a relatively big number of these rare tumors revealed loss of COUP-TFI in all cases except one.

In general, COUP-TFI is recognized as a developmental factor (Tsai and Tsai, 1997), therefore its loss in corticotroph tumors suggest that it may play a role in pituitary differentiation. Corticotroph development is under the control of transcription factors like CUTE, Ptx1, and Lhx3, while the maturation and proliferation of embryonic corticotrophs are subject to the stimulatory action of CRH and the negative feedback of corticosterone. *Ptx1*, which plays a role in the early steps of pituitary organogenesis, is expressed by all early pituitary cells, and only later in development, in synergy with CUTE, is it restricted to the POMC expressing cells (Lamonerie et al., 1996). *Lhx3* is required for the primordial cells to differentiate to pituitary stem cells early in development, but it is not important for the differentiation of the corticotroph lineage. However later in differentiation this factor becomes necessary for the corticotroph cell proliferation (Sheng et al., 1996). CUTE/Neuro D1 is the only factor which seems to be specific for corticotroph cells (Poulin et al., 2000). Recently another factor, the *Tbx19*, was shown to be specifically expressed in primordial corticotroph cells (Liu et al., 2001). However, this factor alone is not able to commit pituitary cells to the corticotroph lineage, and its role in POMC gene expression seems to be controversial.

In the present study it is demonstrated that COUP-TFI is expressed exclusively in ACTH-producing cells of the normal human pituitary but not of corticotrophinomas, and should therefore be considered as a candidate for a role in the differentiation of the corticotroph lineage. Although additional studies should be done before to derive a definite conclusion, its absence from tumoral corticotrophs, which are supposed to be a undifferentiated entity, implies that it plays a role in later differentiation, probably in the final steps of corticotroph development.

These data implicate into pituitary oncogenesis and differentiation a transcription factor previously unrelated to pituitary physiology. COUP-TFI was found to be expressed in the human normal pituitary, and more precisely in ACTH containing cells, but not in any clinically active or inactive corticotroph adenoma. This orphan receptor is known as a developmental factor, therefore its loss upon corticotroph cell transformation suggests that it may be involved in pituitary organogenesis.

SUMMARY

Pituitary adenomas are common neoplasms, with a wide spectrum of pathological presentations. Although they are usually benign they can cause serious clinical problems to the patients bearing them. Studies during the last two decades had focused in elucidating the pathology and molecular biology of these neoplasms. Despite the extensive investigations, the pathogenetic mechanisms that drive the development of these tumors still remain obscure.

This thesis aimed to contribute in this obscure field, providing results about the expression of two candidate tumor suppressor genes in a significant number of pituitary adenomas. The one is ZAC/Zac1, a zinc finger transcription factor which induces cell cycle arrest and apoptosis. We demonstrated that its expression is dramatically reduced/lost in the vast majority of non-functioning pituitary adenomas, a fact that directly implicates this gene in the pathogenesis of this type of tumors. ZAC/Zac1 is not mutated in pituitary tumors, therefore defects in the regulation of its gene expression must be responsible for the low mRNA levels seen in these tumors. In rat ovarian epithelial cells, Zac1 expression is downregulated by the EGFr signaling. However, this model cannot apply for the vast majority of non-functioning tumors, because, as was demonstrated in this study, they do not express EGFr. Another interesting possibility is derived from the recent data about the ZAC promoter. It was found that ZAC is an imprinted gene with a promoter that contains one CpG island. Therefore, one possibility is that promoter hypermethylation may be responsible for the ZAC downregulation in this type of tumors. We showed that treatment of the rat pituitary tumor cell line GH3 with the methylation interfering agent 5-Aza-2´-deoxycytidine results in Zac1 gene expression increase. These promising data imply that similar mechanism may be responsible for the ZAC silencing in the

pituitary tumors. However, to fully address this issue, similar studies must be performed in a human non-functioning pituitary tumor cell model.

The fact that ZAC/Zac1 exerts antiproliferative action in pituitary cells in vitro, gave rise to the question whether the antiproliferative properties of drugs commonly used for the treatment of pituitary tumors can be mediated through this factor. The somatostatin analogue octreotide has been shown to inhibit cell cycle at the G0/G1 transition and this is the point at which ZAC/Zac1 is acting. In this study, it is shown that octreotide is enhancing Zac1 gene expression in vitro, and this effect is pertussis toxin sensitive and is involving PI3K. These data are of particular interest since they provide a novel mechanism through which octreotide can block cell growth. Although the use of octreotide as antiproliferative agent did not give promising results in the treatment of non-functioning adenomas, there is a number of new, more potent, somatostatin analogues, which have to be examined in this context. Elucidating the mechanisms responsible for the regulation of ZAC gene expression will facilitate the development of new pharmaceutical approaches which can target the re-activation of this gene, therefore providing a block for the uncontrolled growth in pituitary tumors that very often cannot be pharmacologically treated.

Another candidate tumor suppressor gene expected to play a role in pituitary tumorigenesis is *MEN1*, the gene responsible for multiple endocrine neoplasia type 1. Originally the studies of *MEN1* gene and gene expression gave disappointing results. *MEN1* gene is not mutated in sporadic pituitary tumors and is normally expressed. However, the lack of appropriate antibodies for *in situ* studies prevented the examination of this factor at protein level. In this study, it is demonstrated, for the first time, the expression pattern of menin in normal and adenomatous pituitary using immunohistochemistry. It is shown that the *MEN1* gene product, menin, is highly expressed in the human adenohypophysis but variably in pituitary tumors. A significant percentage of the tumors studied was found to have very low levels of

menin when compared to the normal adenohypophysis, an observation that implicate *MEN1* in the pathogenesis of sporadic pituitary adenomas not anymore at gene or transcriptional level, but at protein level. Defects in translational or post-translational processing of menin are suspected to be responsible for the low levels of this protein in sporadic pituitary adenomas, although the exact mechanism remains to be elucidated. Nevertheless, the decrease of menin in a significant percentage of pituitary tumors provides a new piece in the complex puzzle of pituitary oncogenesis.

An important concept which could be extrapolated from these results is that the alterations in the levels of ZAC and menin are not result of gene mutation, but of defective gene transcription, as in the case of ZAC, or protein translation/ post-translational modification, as is the case for menin. Our observations are in line with an increasing number of studies, which demonstrate that mutation of a tumor suppressor gene is not as frequent in sporadic cancer as initially expected to be. Different mechanisms of regulation of gene expression, such as promoter hypermethylation, are shown to be often responsible for the silencing of tumor suppressor genes in many forms of human cancer. On the other hand, there are tumor suppressor gene products whose physiological function is secured by a strict regulation at post-translational level. Defective postranslational modifications, such as excessive ubiquitinization, can increase the protein turnover and deprive the cell of this protein and the inhibitory control that it exerts in cell cycle progression. Elucidating the defects in the processing of each tumor suppressor gene product, is of extreme importance since it can suggest new targets for novel, more effective, therapeutical approaches.

The third part of this thesis deals with the transcriptional repressor COUP-TFI. This is the first time that this transcription factor is implicated in pituitary physiology and pathology. Initially described as the key element responsible for the differential response of normal and adenomatous corticotrophs to retinoic acid treatment,

COUP-TFI was soon proven to be a factor restricted to corticotroph cells. We show that COUP-TFI is expressed exclusively in a fraction of the ACTH-producing cells of the normal adenohypophysis, but not in Cushing's associated adenomas. The intriguing finding of highest incidence of COUP-TFI expressing cells in the corticotroph rich area near the residual intermediate lobe, prompted the investigation of this factor in the interesting class of the silent corticotrophinomas. No COUP-TFI was found in most of these rare neoplasms. The finding of loss of COUP-TFI expression in tumoral corticotrophs could implicate it either in growth control or in pituitary differentiation. This factor is known to play an important role in neuronal development, and is considered as a developmental factor. Despite the extensive studies performed, the factors responsible for the differentiation of the corticotroph lineage still remain unidentified. Until now, only the transcription factor CUTE/ NeuroD1 has been demonstrated to be specific for corticotroph differentiation. The fact that COUP-TFI is found exclusively in corticotroph cells but not in any clinically active or silent corticotrophinoma suggest that this factor must be considered as a candidate factor playing a role in pituitary development. Future studies will address whether COUP-TFI is a key element in corticotroph differentiation and it may play a particular role in the physiology of the corticotrophs of the intermediate lobe.

In conclusion, these results highlight the role of these three transcription factors in the pathogenesis of pituitary adenomas, therefore contributing to the understanding of the complex puzzle underlying the formation and maintenance of these peculiar intracranial neoplasms.

Reference List

Abdollahi,A., Bao,R., and Hamilton,T.C. (1999). LOT1 is a growth suppressor gene down-regulated by the epidermal growth factor receptor ligands and encodes a nuclear zinc-finger protein. Oncogene *18*, 6477-6487.

Abdollahi,A., Godwin,A.K., Miller,P.D., Getts,L.A., Schultz,D.C., Taguchi,T., Testa,J.R. , and Hamilton,T.C. (1997). Identification of a gene containing zinc-finger motifs based on lost expression in malignantly transformed rat ovarian surface epithelial cells. Cancer Res. *57*, 2029-2034.

Agarwal,S.K., Guru,S.C., Heppner,C., Erdos,M.R., Collins,R.M., Park,S.Y., Saggar,S., Chandrasekharappa,S.C., Collins,F.S., Spiegel,A.M., Marx,S.J., and Burns,A.L. (1999). Menin interacts with the AP1 transcription factor JunD and represses JunD-activated transcription. Cell *96*, 143-152.

Alexander,J.M., Biller,B.M., Bikkal,H., Zervas,N.T., Arnold,A., and Klibanski,A. (1990). Clinically nonfunctioning pituitary tumors are monoclonal in origin. J. Clin. Invest *86*, 336-340.

Allaerts,W., Carmeliet,P., and Denef,C. (1990). New perspectives in the function of pituitary folliculo-stellate cells. Mol. Cell Endocrinol. *71*, 73-81.

Arima,T., Drewell,R.A., Oshimura,M., Wake,N., and Surani,M.A. (2000). A novel imprinted gene, HYMAI, is located within an imprinted domain on human chromosome 6 containing ZAC. Genomics *67*, 248-255.

Asa,S.L., Somers,K., and Ezzat,S. (1998). The MEN-1 gene is rarely down-regulated in pituitary adenomas. J. Clin. Endocrinol. Metab *83*, 3210-3212.

Bamberger,C.M., Fehn,M., Bamberger,A.M., Ludecke,D.K., Beil,F.U., Saeger,W., and Schulte,H.M. (1999). Reduced expression levels of the cell-cycle inhibitor p27Kip1 in human pituitary adenomas. Eur. J. Endocrinol. *140*, 250-255.

Barlow,D.P. (1995). Gametic imprinting in mammals. Science *270*, 1610-1613.

Bates,A.S., Farrell,W.E., Bicknell,E.J., McNicol,A.M., Talbot,A.J., Broome,J.C., Perrett,C.W., Thakker,R.V., and Clayton,R.N. (1997). Allelic deletion in pituitary adenomas reflects aggressive biological activity and has potential value as a prognostic marker. J. Clin. Endocrinol. Metab *82*, 818-824.

Beck-Peccoz,P., Brucker-Davis,F., Persani,L., Smallridge,R.C., and Weintraub,B.D. (1996). Thyrotropin-secreting pituitary tumors. Endocr. Rev. *17*, 610-638.

Bilanges,B., Varrault,A., Basyuk,E., Rodriguez,C., Mazumdar,A., Pantaloni,C., Bockaert,J., Theillet,C., Spengler,D., and Journot,L. (1999). Loss of expression of the candidate tumor suppressor gene ZAC in breast cancer cell lines and primary tumors. Oncogene *18*, 3979-3988.

Bird,A. (1992). The essentials of DNA methylation. Cell *70*, 5-8.

Birman,P., Michard,M., Li,J.Y., Peillon,F., and Bression,D. (1987). Epidermal growth factor-binding sites, present in normal human and rat pituitaries, are absent in human pituitary adenomas. J. Clin. Endocrinol. Metab *65*, 275-281.

Boggild,M.D., Jenkinson,S., Pistorello,M., Boscaro,M., Scanarini,M., McTernan,P., Perrett,C.W., Thakker,R.V., and Clayton,R.N. (1994). Molecular genetic studies of sporadic pituitary tumors. J. Clin. Endocrinol. Metab *78*, 387-392.

Boscaro,M., Barzon,L., Fallo,F., and Sonino,N. (2001). Cushing' s syndrome. Lancet*357*, 783-791.

Buckley,N., Bates,A.S., Broome,J.C., Strange,R.C., Perrett,C.W., Burke,C.W., and Clayton,R.N. (1995). P53 protein accumulates in Cushings adenomas and invasive non- functional adenomas. J. Clin. Endocrinol. Metab *80*, 4.

Caccavelli,L., Feron,F., Morange,I., Rouer,E., Benarous,R., Dewailly,D., Jaquet,P., Kordon,C., and Enjalbert,A. (1994). Decreased expression of the two D2 dopamine receptor isoforms in bromocriptine-resistant prolactinomas. Neuroendocrinology *60*, 314-322.

Cetani,F., Pardi,E., Cianferotti,L., Vignali,E., Picone,A., Miccoli,P., Pinchera,A., and Marcocci,C. (1999). A new mutation of the MEN1 gene in an italian kindred with multiple endocrine neoplasia type 1. Eur. J. Endocrinol. *140*, 429-433.

Chabot,J.G., Walker,P., and Pelletier,G. (1986). Distribution of epidermal growth factor binding sites in the adult rat anterior pituitary gland. Peptides *7*, 45-50.

Chaidarun,S.S., Eggo,M.C., Sheppard,M.C., and Stewart,P.M. (1994). Expression of epidermal growth factor (EGF), its receptor, and related oncoprotein (erbB-2) in human pituitary tumors and response to EGF in vitro. Endocrinology *135*, 2012-2021.

Chandrasekharappa,S.C., Guru,S.C., Manickam,P., Olufemi,S.E., Collins,F.S., Emmert-Buck,M.R., Debelenko,L.V., Zhuang,Z., Lubensky,I.A., Liotta,L.A., Crabtree,J.S., Wang,Y., Roe,B.A., Weisemann,J., Boguski,M.S., Agarwal,S.K., Kester,M.B., Kim,Y.S., Heppner,C., Dong,Q., Spiegel,A.M., Burns,A.L., and Marx,S.J. (1997). Positional cloning of the gene for multiple endocrine neoplasia-type 1. Science *276*, 404-407.

Chen,L., Fitzpatrick,V.D., Vandlen,R.L., and Tashjian,A.H., Jr. (1997). Both overlapping and distinct signaling pathways for somatostatin receptor subtypes SSTR1 and SSTR2 in pituitary cells. J. Biol. Chem. *272*, 18666-18672.

Cheung,N.W. and Boyages,S.C. (1995). Somatostatin-14 and its analog octreotide exert a cytostatic effect on GH3 rat pituitary tumor cell proliferation via a transient G0/G1 cell cycle block. Endocrinology *136*, 4174-4181.

Childs,G.V., Rougeau,D., and Unabia,G. (1995). Corticotropin-releasing hormone and epidermal growth factor: mitogens for anterior pituitary corticotropes. Endocrinology *136*, 1595-1602.

Clayton,R.N., Pfeifer,M., Atkinson,A.B., Belchetz,P., Wass,J.A., Kyrodimou,E., Vanderpump,M., Simpson,D., Bicknell,J., and Farrell,W.E. (2000). Different patterns of allelic loss (loss of heterozygosity) in recurrent human pituitary tumors provide evidence for multiclonal origins. Clin. Cancer Res. *6*, 3973-3982.

Colao,A., Cerbone,G., Cappabianca,P., Ferone,D., Alfieri,A., Di Salle,F., Faggiano,A., Merola,B., de Divitiis,E., and Lombardi,G. (1998). Effect of surgery and radiotherapy on visual and endocrine function in nonfunctioning pituitary adenomas. J. Endocrinol. Invest *21*, 284-290.

Colao,A., Di Sarno,A., Marzullo,P., Di Somma,C., Cerbone,G., Landi,M.L., Faggiano,A., Merola,B., and Lombardi,G. (2000). New medical approaches in pituitary adenomas. Horm. Res. *53 Suppl 3:76-87.*, 76-87.

Colao,A., Di Sarno,A., Sarnacchiaro,F., Ferone,D., Di Renzo,G., Merola,B., Annunziato,L. , and Lombardi,G. (1997). Prolactinomas resistant to standard dopamine agonists respond to chronic cabergoline treatment. J. Clin. Endocrinol. Metab *82*, 876-883.

Cooney,A.J., Leng,X., Tsai,S.Y., O' Malley,B.W., and Tsai,M.J. (1993). Multiple mechanisms of chicken ovalbumin upstream promoter transcription factor-dependent repression of transactivation by the vitamin D, thyroid hormone, and retinoic acid receptors. J. Biol. Chem. *268*, 4152-4160.

Cooper,D.N. and Krawczak,M. (1989). Cytosine methylation and the fate of CpG dinucleotides in vertebrate genomes. Hum. Genet. *83*, 181-188.

Dahia,P.L., Aguiar,R.C., Honegger,J., Fahlbush,R., Jordan,S., Lowe,D.G., Lu,X., Clayton,R.N., Besser,G.M., and Grossman,A.B. (1998). Mutation and expression analysis of the p27/kip1 gene in corticotrophin- secreting tumours. Oncogene *16*, 69-76.

Dahia,P.L. and Grossman,A.B. (1999). The molecular pathogenesis of corticotroph tumors. Endocr. Rev. *20*, 136-155.

Downward,J., Yarden,Y., Mayes,E., Scrace,G., Totty,N., Stockwell,P., Ullrich,A., Schlessinger,J., and Waterfield,M.D. (1984). Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature *307*, 521-527.

El Kharroubi,A., Piras,G., and Stewart,C.L. (2001). DNA demethylation reactivates a subset of imprinted genes in uniparental mouse embryonic fibroblasts. J. Biol. Chem. *276*, 8674-8680.

Eriksson,B. and Oberg,K. (1999). Summing up 15 years of somatostatin analog therapy in neuroendocrine tumors: future outlook. Ann. Oncol. *10 Suppl 2:S31-8.*, S31-S38.

Fan,X. and Childs,G.V. (1995). Epidermal growth factor and transforming growth factor-alpha messenger ribonucleic acids and their receptors in the rat anterior pituitary: localization and regulation. Endocrinology *136*, 2284-2293.

Farrell,W.E., Simpson,D.J., Bicknell,J., Magnay,J.L., Kyrodimou,E., Thakker,R.V., and Clayton,R.N. (1999). Sequence analysis and transcript expression of the MEN1 gene in sporadic pituitary tumours. Br. J. Cancer *80*, 44-50.

Farrell,W.E., Simpson,D.J., Bicknell,J.E., Talbot,A.J., Bates,A.S., and Clayton,R.N. (1997). Chromosome 9p deletions in invasive and noninvasive nonfunctional pituitary adenomas: the deleted region involves markers outside of the MTS1 and MTS2 genes. Cancer Res. *57*, 2703-2709.

Fero,M.L., Randel,E., Gurley,K.E., Roberts,J.M., and Kemp,C.J. (1998). The murine gene p27Kip1 is haplo-insufficient for tumour suppression. Nature *396*, 177-180.

Fero,M.L., Rivkin,M., Tasch,M., Porter,P., Carow,C.E., Firpo,E., Polyak,K., Tsai,L.H., Broudy,V., Perlmutter,R.M., Kaushansky,K., and Roberts,J.M. (1996). A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. Cell *85*, 733-744.

Ferro,T., D' Agostino,D.M., Barzon,L., Theodoropoulou,M., Rosato.A., Esposito.G., Cavallari,I., Pasquali,C., Boscaro,M., Pagotto,U., Chieco-Bianchi,L., Fallo,F. and Ciminale,V. In situ analysis of Menin expression using a monoclonal antibody and laser scanning microscopy. *Manuscript submitted.*

Foulkes,W.D., Ragoussis,J., Stamp,G.W., Allan,G.J., and Trowsdale,J. (1993). Frequent loss of heterozygosity on chromosome 6 in human ovarian carcinoma. Br. J. Cancer *67*, 551-559.

Franklin,D.S., Godfrey,V.L., Lee,H., Kovalev,G.I., Schoonhoven,R., Chen-Kiang,S., Su,L., and Xiong,Y. (1998). CDK inhibitors p18(INK4c) and p27(Kip1) mediate two separate pathways to collaboratively suppress pituitary tumorigenesis. Genes Dev. *12*, 2899-2911.

Frost,S.J., Simpson,D.J., Clayton,R.N., and Farrell,W.E. (1999). Transfection of an inducible p16/CDKN2A construct mediates reversible growth inhibition and G1 arrest in the AtT20 pituitary tumor cell line. Mol. Endocrinol. *13*, 1801-1810.

Fujii,H., Szumel,R., Marsh,C., Zhou,W., and Gabrielson,E. (1996). Genetic progression, histological grade, and allelic loss in ductal carcinoma in situ of the breast. Cancer Res. *56*, 5260-5265.

Greenman,Y. and Melmed,S. (1994b). Expression of three somatostatin receptor subtypes in pituitary adenomas: evidence for preferential SSTR5 expression in the mammosomatotroph lineage. J. Clin. Endocrinol. Metab *79*, 724-729.

Greenman,Y. and Melmed,S. (1994a). Heterogeneous expression of two somatostatin receptor subtypes in pituitary tumors. J. Clin. Endocrinol. Metab *78*, 398-403.

Gu,Y.Z. and Schonbrunn,A. (1997). Coupling specificity between somatostatin receptor sst2A and G proteins: isolation of the receptor-G protein complex with a receptor antibody . Mol. Endocrinol. *11*, 527-537.

Guru,S.C., Goldsmith,P.K., Burns,A.L., Marx,S.J., Spiegel,A.M., Collins,F.S., and Chandrasekharappa,S.C. (1998). Menin, the product of the MEN1 gene, is a nuclear protein. Proc. Natl. Acad. Sci. U. S. A *95*, 1630-1634.

Hardy J (1979). Transphenoidal microsurgical treatment of pituitary tumours. In Recent advances in the diagnosis and treatment of pituitary tumours, Linfoot J, ed. Raven Press, New York, pp. 375-388.

Harvey,M., Vogel,H., Lee,E.Y., Bradley,A., and Donehower,L.A. (1995). Mice deficient in both p53 and Rb develop tumors primarily of endocrine origin. Cancer Res. *55*, 1146-1151.

Hayward,B.E., Barlier,A., Korbonits,M., Grossman,A.B., Jacquet,P., Enjalbert,A., and Bonthron,D.T. (2001). Imprinting of the G(s)alpha gene GNAS1 in the pathogenesis of acromegaly. J. Clin. Invest *107*, R31-R36.

Heaney,A.P., Horwitz,G.A., Wang,Z., Singson,R., and Melmed,S. (1999). Early involvement of estrogen-induced pituitary tumor transforming gene and fibroblast growth factor expression in prolactinoma pathogenesis. Nat. Med. *5*, 1317-1321.

Heaney,A.P., Singson,R., McCabe,C.J., Nelson,V., Nakashima,M., and Melmed,S. (2000). Expression of pituitary-tumour transforming gene in colorectal tumours. Lancet *355*, 716-719.

Heppner,C., Bilimoria,K.Y., Agarwal,S.K., Kester,M., Whitty,L.J., Guru,S.C., Chandrasekharappa,S.C., Collins,F.S., Spiegel,A.M., Marx,S.J., and Burns,A.L. (2001). The tumor suppressor protein menin interacts with NF-kappaB proteins and inhibits NF-kappaB-mediated transactivation. Oncogene *20*, 4917-4925.

Heppner,C., Kester,M.B., Agarwal,S.K., Debelenko,L.V., Emmert-Buck,M.R., Guru,S.C., Manickam,P., Olufemi,S.E., Skarulis,M.C., Doppman,J.L., Alexander,R.H., Kim,Y.S., Saggar,S.K., Lubensky,I.A., Zhuang,Z., Liotta,L.A., Chandrasekharappa,S.C., Collins,F.S., Spiegel,A.M., Burns,A.L., and Marx,S.J. (1997). Somatic mutation of the MEN1 gene in parathyroid tumours. Nat. Genet. *16*, 375-378.

Herman,J.G. (1999). Hypermethylation of tumor suppressor genes in cancer. Semin. Cancer Biol. *9*, 359-367.

Hibberts,N.A., Simpson,D.J., Bicknell,J.E., Broome,J.C., Hoban,P.R., Clayton,R.N., and Farrell,W.E. (1999). Analysis of cyclin D1 (CCND1) allelic imbalance and overexpression in sporadic human pituitary tumors. Clin. Cancer Res. *5*, 2133-2139.

Hofland,L.J., Visser-Wisselaar,H.A., and Lamberts,S.W. (1995). Somatostatin analogs: clinical application in relation to human somatostatin receptor subtypes. Biochem. Pharmacol. *50*, 287-297.

Hollstein,M., Sidransky,D., Vogelstein,B., and Harris,C.C. (1991). p53 mutations in human cancers. Science *253*, 49-53.

Holm,R. (1995). Null cell adenomas and oncocytomas of the pituitary gland. Pathol. Res. Pract. *191*, 348-352.

Horvath,E., Kovacs,K., Killinger,D.W., Smyth,H.S., Platts,M.E., and Singer,W. (1980). Silent corticotropic adenomas of the human pituitary gland: a histologic, immunocytologic, and ultrastructural study. Am. J. Pathol. *98*, 617-638.

Hu,N., Gutsmann,A., Herbert,D.C., Bradley,A., Lee,W.H., and Lee,E.Y. (1994). Heterozygous Rb-1 delta 20/+mice are predisposed to tumors of the pituitary gland with a nearly complete penetrance. Oncogene *9*, 1021-1027.

Ikeda,H., Yoshimoto,T., and Shida,N. (1997). Molecular analysis of p21 and p27 genes in human pituitary adenomas. Br. J. Cancer *76*, 1119-1123.

Ikeo,Y., Sakurai,A., Suzuki,R., Zhang,M.X., Koizumi,S., Takeuchi,Y., Yumita,W., Nakayama,J., and Hashizume,K. (2000). Proliferation-associated expression of the MEN1 gene as revealed by in situ hybridization: possible role of the menin as a negative regulator of cell proliferation under DNA damage. Lab Invest *80*, 797-804.

Jacks,T., Fazeli,A., Schmitt,E.M., Bronson,R.T., Goodell,M.A., and Weinberg,R.A. (1992). Effects of an Rb mutation in the mouse. Nature *359*, 295-300.

Jaffrain-Rea,M.L., Petrangeli,E., Lubrano,C., Minniti,G., Di Stefano,D., Sciarra,F., Frati,L., Tamburrano,G., Cantore,G., and Gulino,A. (1998). Epidermal growth factor binding sites in human pituitary macroadenomas. J. Endocrinol. *158*, 425-433.

Jin,L., Qian,X., Kulig,E., Sanno,N., Scheithauer,B.W., Kovacs,K., Young,W.F., Jr., and Lloyd,R.V. (1997). Transforming growth factor-beta, transforming growth factor-beta receptor II, and p27Kip1 expression in nontumorous and neoplastic human pituitaries. Am. J. Pathol. *151*, 509-519.

Jones,P.A. (1996). DNA methylation errors and cancer. Cancer Res. *56*, 2463-2467.

Jonk,L.J., de Jonge,M.E., Pals,C.E., Wissink,S., Vervaart,J.M., Schoorlemmer,J., and Kruijer,W. (1994). Cloning and expression during development of three murine members of the COUP family of nuclear orphan receptors. Mech. Dev. *47*, 81-97.

Kaji,H., Canaff,L., Goltzman,D., and Hendy,G.N. (1999). Cell cycle regulation of menin expression. Cancer Res. *59*, 5097-5101.

Kaji,H., Canaff,L., Lebrun,J.J., Goltzman,D., and Hendy,G.N. (2001). Inactivation of menin, a Smad3-interacting protein, blocks transforming growth factor type beta signaling. Proc. Natl. Acad. Sci. U. S. A *98*, 3837-3842.

Kamiya,M., Judson,H., Okazaki,Y., Kusakabe,M., Muramatsu,M., Takada,S., Takagi,N., Arima,T., Wake,N., Kamimura,K., Satomura,K., Hermann,R., Bonthron,D.T., and Hayashizaki,Y. (2000). The cell cycle control gene ZAC/PLAGL1 is imprinted--a strong candidate gene for transient neonatal diabetes. Hum. Mol. Genet. *9*, 453-460.

Kim,Y.S., Burns,A.L., Goldsmith,P.K., Heppner,C., Park,S.Y., Chandrasekharappa,S.C., Collins,F.S., Spiegel,A.M., and Marx,S.J. (1999). Stable overexpression of MEN1 suppresses tumorigenicity of RAS. Oncogene *18*, 5936-5942.

Kioussi,C., Carriere,C., and Rosenfeld,M.G. (1999). A model for the development of the hypothalamic-pituitary axis: transcribing the hypophysis. Mech. Dev. *81*, 23-35.

Kiyokawa,H., Kineman,R.D., Manova-Todorova,K.O., Soares,V.C., Hoffman,E.S., Ono,M., Khanam,D., Hayday,A.C., Frohman,L.A., and Koff,A. (1996). Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). Cell *85*, 721-732.

Kliewer,S.A., Umesono,K., Heyman,R.A., Mangelsdorf,D.J., Dyck,J.A., and Evans,R.M. (1992). Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling. Proc. Natl. Acad. Sci. U. S. A *89*, 1448-1452.

Klijn,J.G., Berns,P.M., Schmitz,P.I., and Foekens,J.A. (1992). The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. Endocr. Rev. *13*, 3-17.

Knudson,A.G., Jr. (1975). The genetics of childhood cancer. Cancer *35*, 1022-1026.

Kontogeorgos,G., Kapranos,N., Thodou,E., Sambaziotis,D., and Tsagarakis,S. (1999). Immunocytochemical accumulation of p53 in corticotroph adenomas: relationship with heat shock proteins and apoptosis. Pituitary. *1*, 207-212.

Ladias,J.A. and Karathanasis,S.K. (1991). Regulation of the apolipoprotein AI gene by ARP-1, a novel member of the steroid receptor superfamily. Science *251*, 561-565.

Lamberts,S.W., de Lange,S.A., and Stefanko,S.Z. (1982). Adrenocorticotropin-secreting pituitary adenomas originate from the anterior or the intermediate lobe in Cushing' s disease: differences in the regulation of hormone secretion. J. Clin. Endocrinol. Metab *54*, 286-291.

Lamberts,S.W., Krenning,E.P., and Reubi,J.C. (1991). The role of somatostatin and its analogs in the diagnosis and treatment of tumors. Endocr. Rev. *12*, 450-482.

Lamberts,S.W., van der Lely,A.J., de Herder,W.W., and Hofland,L.J. (1996). Octreotide. N. Engl. J. Med. *334*, 246-254.

Lamonerie,T., Tremblay,J.J., Lanctot,C., Therrien,M., Gauthier,Y., and Drouin,J. (1996). Ptx1, a bicoid-related homeo box transcription factor involved in transcription of the pro-opiomelanocortin gene. Genes Dev. *10*, 1284-1295.

Landis,C.A., Masters,S.B., Spada,A., Pace,A.M., Bourne,H.R., and Vallar,L. (1989). GTPase inhibiting mutations activate the alpha chain of Gs and stimulate adenylyl cyclase in human pituitary tumours. Nature *340*, 692-696.

Larsson,C., Skogseid,B., Oberg,K., Nakamura,Y., and Nordenskjold,M. (1988). Multiple endocrine neoplasia type 1 gene maps to chromosome 11 and is lost in insulinoma. Nature *332*, 85-87.

Lee,J.T. and Jaenisch,R. (1997). The (epi)genetic control of mammalian X-chromosome inactivation. Curr. Opin. Genet. Dev. *7*, 274-280.

Lemmens,I., Van de Ven,W.J., Kas,K., Zhang,C.X., Giraud,S., Wautot,V., Buisson,N., De Witte,K., Salandre,J., Lenoir,G., Pugeat,M., Calender,A., Parente,F., Quincey,D., Gaudray,P., De Wit,M.J., Lips,C.J., Hoppener,J.W., Khodaei,S., Grant,A.L., Weber,G., Kytola,S., Teh,B.T., Farnebo,F., Thakker,R.V. (1997). Identification of the multiple endocrine neoplasia type 1 (MEN1) gene. The European Consortium on MEN1. Hum. Mol. Genet. *6*, 1177-1183.

LeRiche,V.K., Asa,S.L., and Ezzat,S. (1996). Epidermal growth factor and its receptor (EGF-R) in human pituitary adenomas: EGF-R correlates with tumor aggressiveness. J. Clin. Endocrinol. Metab *81*, 656-662.

Lidhar,K., Korbonits,M., Jordan,S., Khalimova,Z., Kaltsas,G., Lu,X., Clayton,R.N., Jenkins,P.J., Monson,J.P., Besser,G.M., Lowe,D.G., and Grossman,A.B. (1999). Low expression of the cell cycle inhibitor p27Kip1 in normal corticotroph cells, corticotroph tumors, and malignant pituitary tumors. J. Clin. Endocrinol. Metab *84*, 3823-3830.

Liu,J., Lin,C., Gleiberman,A., Ohgi,K.A., Herman,T., Huang,H.P., Tsai,M.J., and Rosenfeld,M.G. (2001). Tbx19, a tissue-selective regulator of POMC gene expression. Proc. Natl. Acad. Sci. U. S. A *98*, 8674-8679.

Lloyd,R.V., Jin,L., Fields,K., Chandler,W.F., Horvath,E., Stefaneanu,L., and Kovacs,K. (1991). Analysis of pituitary hormones and chromogranin A mRNAs in null cell adenomas, oncocytomas, and gonadotroph adenomas by in situ hybridization. Am. J. Pathol. *139*, 553-564.

Lloyd,R.V., Jin,L., Qian,X., and Kulig,E. (1997). Aberrant p27kip1 expression in endocrine and other tumors. Am. J. Pathol. *150*, 401-407.

Loda,M., Cukor,B., Tam,S.W., Lavin,P., Fiorentino,M. , Draetta,G.F., Jessup,J.M., and Pagano,M. (1997). Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. Nat. Med. *3*, 231-234.

Losa,M., Ciccarelli,E., Mortini,P., Barzaghi,R., Gaia,D., Faccani,G., Papotti,M., Mangili,F., Terreni,M.R., Camanni,F., and Giovanelli,M. (2001). Effects of octreotide treatment on the proliferation and apoptotic index of GH-secreting pituitary adenomas. J. Clin. Endocrinol. Metab *86*, 5194-5200.

Marx,S.J., Agarwal,S.K., Kester,M.B., Heppner,C., Kim,Y.S., Skarulis,M.C., James,L.A., Goldsmith,P.K., Saggar,S.K., Park,S.Y., Spiegel,A.M., Burns,A.L., Debelenko,L.V., Zhuang,Z., Lubensky,I.A., Liotta,L.A. , Emmert-Buck,M.R., Guru,S.C., Manickam,P., Crabtree,J., Erdos,M.R., Collins,F.S., and Chandrasekharappa,S.C. (1999). Multiple endocrine neoplasia type 1: clinical and genetic features of the hereditary endocrine neoplasias. Recent Prog. Horm. Res. *54:397-438; discussion 438-9.*, 397-438.

Melmed,S., Jackson,I., Kleinberg,D., and Klibanski,A. (1998). Current treatment guidelines for acromegaly. J. Clin. Endocrinol. Metab *83*, 2646-2652.

Mezey,E., Hunyady,B., Mitra,S., Hayes,E., Liu,Q., Schaeffer,J., and Schonbrunn,A. (1998). Cell specific expression of the sst2A and sst5 somatostatin receptors in the rat anterior pituitary. Endocrinology *139*, 414-419.

Miyajima,N., Kadowaki,Y., Fukushige,S., Shimizu,S., Semba,K., Yamanashi,Y., Matsubara,K. , Toyoshima,K., and Yamamoto,T. (1988). Identification of two novel members of erbA superfamily by molecular cloning: the gene products of the two are highly related to each other. Nucleic Acids Res. *16*, 11057-11074.

Nakayama,K., Ishida,N., Shirane,M., Inomata,A., Inoue,T., Shishido,N., Horii,I., Loh,D.Y., and Nakayama,K. (1996). Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell *85*, 707-720.

Ohkura,N., Kishi,M., Tsukada,T., and Yamaguchi,K. (2001). Menin, a gene product responsible for multiple endocrine neoplasia type 1, interacts with the putative tumor metastasis suppressor nm23. Biochem. Biophys. Res. Commun. *282*, 1206-1210.

Paez-Pereda,M., Kovalovsky,D., Hopfner,U., Theodoropoulou,M., Pagotto,U., Uhl,E., Losa,M., Stalla,J., Grubler,Y., Missale,C., Arzt,E., and Stalla,G.K. (2001). Retinoic acid prevents experimental Cushing syndrome. J. Clin. Invest *108*, 1123-1131.

Pagano,M., Tam,S.W., Theodoras,A.M., Beer-Romero,P., Del Sal,G., Chau,V., Yew,P.R., Draetta,G.F., and Rolfe,M. (1995). Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science *269*, 682-685.

Pages,P., Benali,N., Saint-Laurent,N., Esteve,J.P., Schally,A.V., Tkaczuk,J., Vaysse,N., Susini,C., and Buscail,L. (1999). sst2 somatostatin receptor mediates cell cycle arrest and induction of p27(Kip1). Evidence for the role of SHP-1. J. Biol. Chem. *274*, 15186-15193.

Pagotto,U., Arzberger,T., Ciani,E., Lezoualc' h,F., Pilon,C., Journot,L., Spengler,D., and Stalla,G.K. (1999). Inhibition of Zac1, a new gene differentially expressed in the anterior pituitary, increases cell proliferation. Endocrinology *140*, 987-996.

Pagotto,U., Arzberger,T., Theodoropoulou,M., Grubler,Y., Pantaloni,C., Saeger,W., Losa,M., Journot,L., Stalla,G.K., and Spengler,D. (2000). The expression of the antiproliferative gene ZAC is lost or highly reduced in nonfunctioning pituitary adenomas. Cancer Res. *60*, 6794-6799.

Pannett,A.A. and Thakker,R.V. (1999). Multiple endocrine neoplasia type 1. Endocr. Relat Cancer *6*, 449-473.

Patel,Y.C., Greenwood,M., Kent,G., Panetta,R., and Srikant,C.B. (1993). Multiple gene transcripts of the somatostatin receptor SSTR2: tissue selective distribution and cAMP regulation. Biochem. Biophys. Res. Commun. *192*, 288-294.

Patel,Y.C., Greenwood,M.T., Panetta,R., Demchyshyn,L., Niznik,H., and Srikant,C.B. (1995). The somatostatin receptor family. Life Sci. *57*, 1249-1265.

Pei,L. (2001). Identification of c-myc as a down-stream target for pituitary tumor- transforming gene. J. Biol. Chem. *276*, 8484-8491.

Pei,L. and Melmed,S. (1997). Isolation and characterization of a pituitary tumor-transforming gene (PTTG). Mol. Endocrinol. *11*, 433-441.

Pei,L., Melmed,S., Scheithauer,B., Kovacs,K., Benedict,W.F., and Prager,D. (1995). Frequent loss of heterozygosity at the retinoblastoma susceptibility gene (RB) locus in aggressive pituitary tumors: evidence for a chromosome 13 tumor suppressor gene other than RB. Cancer Res. *55*, 1613-1616.

Pereira,F.A., Qiu,Y., Tsai,M.J., and Tsai,S.Y. (1995). Chicken ovalbumin upstream promoter transcription factor (COUP-TF): expression during mouse embryogenesis. J. Steroid Biochem. Mol. Biol. *53*, 503-508.

Pereira,F.A., Tsai,M.J., and Tsai,S.Y. (2000). COUP-TF orphan nuclear receptors in development and differentiation. Cell Mol. Life Sci. *57*, 1388-1398.

Pestell,R.G., Albanese,C., Reutens,A.T., Segall,J.E., Lee,R.J., and Arnold,A. (1999). The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation. Endocr. Rev. *20*, 501-534.

Piras,G., El Kharroubi,A., Kozlov,S., Escalante-Alcalde,D., Hernandez,L., Copeland,N.G., Gilbert,D.J., Jenkins,N.A., and Stewart,C.L. (2000). Zac1 (Lot1), a potential tumor suppressor gene, and the gene for epsilon-sarcoglycan are maternally imprinted genes: identification by a subtractive screen of novel uniparental fibroblast lines. Mol. Cell Biol. *20*, 3308-3315.

Poulin,G., Lebel,M., Chamberland,M., Paradis,F.W., and Drouin,J. (2000). Specific protein-protein interaction between basic helix-loop-helix transcription factors and homeoproteins of the Pitx family. Mol. Cell Biol. *20*, 4826-4837.

Prezant,T.R., Levine,J., and Melmed,S. (1998). Molecular characterization of the men1 tumor suppressor gene in sporadic pituitary tumors. J. Clin. Endocrinol. Metab *83*, 1388-1391.

Qiu,Y., Cooney,A.J., Kuratani,S., DeMayo,F.J., Tsai,S.Y., and Tsai,M.J. (1994). Spatiotemporal expression patterns of chicken ovalbumin upstream promoter-transcription factors in the developing mouse central nervous system: evidence for a role in segmental patterning of the diencephalon. Proc. Natl. Acad. Sci. U. S. A *91*, 4451-4455.

Qiu,Y., Pereira,F.A., DeMayo,F.J., Lydon,J.P., Tsai,S.Y., and Tsai,M.J. (1997). Null mutation of mCOUP-TFI results in defects in morphogenesis of the glossopharyngeal ganglion, axonal projection, and arborization. Genes Dev. *11*, 1925-1937.

Ray,D. and Melmed,S. (1997). Pituitary cytokine and growth factor expression and action. Endocr. Rev. *18*, 206-228.

Renner,U., Pagotto,U., Arzt,E., and Stalla,G.K. (1996). Autocrine and paracrine roles of polypeptide growth factors, cytokines and vasogenic substances in normal and tumorous pituitary function and growth: a review. Eur. J. Endocrinol. *135*, 515-532.

Ritchie,H.H., Wang,L.H., Tsai,S., O' Malley,B.W., and Tsai,M.J. (1990). COUP-TF gene: a structure unique for the steroid/thyroid receptor superfamily. Nucleic Acids Res. *18*, 6857-6862.

Saez,C., Japon,M.A., Ramos-Morales,F., Romero,F., Segura,D.I., Tortolero,M., and Pintor-Toro,J.A. (1999). hpttg is over-expressed in pituitary adenomas and other primary epithelial neoplasias. Oncogene *18*, 5473-5476.

Sagami,I., Tsai,S.Y., Wang,H., Tsai,M.J., and O' Malley,B.W. (1986). Identification of two factors required for transcription of the ovalbumin gene. Mol. Cell Biol. *6*, 4259-4267.

Satta,M.A., Korbonits,M., Jacobs,R.A., Bolden-Dwinfour,D.A., Kaltsas,G.A., Vangeli,V., Adams,E., Fahlbusch,R., and Grossman,A.B. (1999). Expression of menin gene mRNA in pituitary tumours. Eur. J. Endocrinol. *140*, 358-361.

Scheithauer,B.W., Jaap,A.J., Horvath,E., Kovacs,K., Lloyd,R.V., Meyer,F.B., Laws,E.R., Jr., and Young,W.F., Jr. (2000). Clinically silent corticotroph tumors of the pituitary gland. Neurosurgery *47*, 723-729.

Scheithauer,B.W., Kovacs,K., and Horvath,E. (1996). The adenohypophysis. In Bloodworth' s Endocrine Pathology, Bloodworth J.M.B., Lechango J, and J.P.Grande, eds. Williams & Wilkins), pp. 85-145.

Schlegel,J., Merdes,A., Stumm,G., Albert,F.K., Forsting,M., Hynes,N., and Kiessling,M. (1994). Amplification of the epidermal-growth-factor-receptor gene correlates with different growth behaviour in human glioblastoma. Int. J. Cancer *56*, 72-77.

Schmidt,M.C., Henke,R.T., Stangl,A.P., Meyer-Puttlitz,B., Stoffel-Wagner,B., Schramm,J., and von Deimling,A. (1999). Analysis of the MEN1 gene in sporadic pituitary adenomas. J. Pathol. *188*, 168-173.

Schwartz, J. and Cherny, R. (1992). Intercellular communication within the anterior pituitary influencing the secretion of hypophysial hormones. Endocr. Rev. *13*, 453-475.

Sheng,H.Z., Zhadanov,A.B., Mosinger,B., Jr., Fujii,T., Bertuzzi,S., Grinberg,A., Lee,E.J., Huang,S.P., Mahon,K.A., and Westphal,H. (1996). Specification of pituitary cell lineages by the LIM homeobox gene Lhx3. Science *272*, 1004-1007.

Shimon,I., Taylor,J.E., Dong,J.Z., Bitonte,R.A., Kim,S., Morgan,B., Coy,D.H., Culler,M.D., and Melmed,S. (1997a). Somatostatin receptor subtype specificity in human fetal pituitary cultures. Differential role of SSTR2 and SSTR5 for growth hormone, thyroid-stimulating hormone, and prolactin regulation. J. Clin. Invest *99*, 789-798.

Shimon,I., Yan,X., Taylor,J.E., Weiss,M.H., Culler,M.D., and Melmed,S. (1997b). Somatostatin receptor (SSTR) subtype-selective analogues differentially suppress in vitro growth hormone and prolactin in human pituitary adenomas. Novel potential therapy for functional pituitary tumors. J. Clin. Invest *100*, 2386-2392.

Simon,M.I., Strathmann,M.P., and Gautam,N. (1991). Diversity of G proteins in signal transduction. Science *252*, 802-808.

Simpson,D.J., Bicknell,J.E., McNicol,A.M., Clayton,R.N., and Farrell,W.E. (1999b). Hypermethylation of the p16/CDKN2A/MTSI gene and loss of protein expression is associated with nonfunctional pituitary adenomas but not somatotrophinomas. Genes Chromosomes. Cancer *24*, 328-336.

Simpson,D.J., Hibberts,N.A., McNicol,A.M., Clayton,R.N., and Farrell,W.E. (2000). Loss of pRb expression in pituitary adenomas is associated with methylation of the RB1 CpG island. Cancer Res. *60*, 1211-1216.

Simpson,D.J., Magnay,J., Bicknell,J.E., Barkan,A.L., McNicol,A.M., Clayton,R.N., and Farrell,W.E. (1999a). Chromosome 13q deletion mapping in pituitary tumors: infrequent loss of the retinoblastoma susceptibility gene (RB1) locus despite loss of RB1 protein product in somatotrophinomas. Cancer Res. *59*, 1562-1566.

Snyder,P.J. (1995). Extensive personal experience: gonadotroph adenomas. J. Clin. Endocrinol. Metab *80*, 1059-1061.

Sonino,N., Zielezny,M., Fava,G.A., Fallo,F., and Boscaro,M. (1996). Risk factors and long-term outcome in pituitary-dependent Cushing' s disease. J. Clin. Endocrinol. Metab*81*, 2647-2652.

Spengler,D., Villalba,M., Hoffmann,A., Pantaloni,C., Houssami,S., Bockaert,J., and Journot,L. (1997). Regulation of apoptosis and cell cycle arrest by Zac1, a novel zinc finger protein expressed in the pituitary gland and the brain. EMBO J. *16*, 2814-2825.

Stewart,P.M. (2000). Current therapy for acromegaly. Trends Endocrinol. Metab *11*, 128-132.

Suzuki,T., Moriya,T., Darnel,A.D., Takeyama,J., and Sasano,H. (2000). Immunohistochemical distribution of chicken ovalbumin upstream promoter transcription factor II in human tissues. Mol. Cell Endocrinol. *164*, 69-75.

Taguchi,T., Jhanwar,S.C., Siegfried,J.M., Keller,S.M., and Testa,J.R. (1993). Recurrent deletions of specific chromosomal sites in 1p, 3p, 6q, and 9p in human malignant mesothelioma. Cancer Res. *53*, 4349-4355.

Takeuchi,S., Koeffler,H.P., Hinton,D.R., Miyoshi,I., Melmed,S., and Shimon,I. (1998). Mutation and expression analysis of the cyclin-dependent kinase inhibitor gene p27/Kip1 in pituitary tumors. J. Endocrinol. *157*, 337-341.

Tanaka,C., Kimura,T., Yang,P., Moritani,M., Yamaoka,T., Yamada,S., Sano,T., Yoshimoto,K., and Itakura,M. (1998). Analysis of loss of heterozygosity on chromosome 11 and infrequent inactivation of the MEN1 gene in sporadic pituitary adenomas. J. Clin. Endocrinol. Metab *83*, 2631-2634.

Thapar,K., Scheithauer,B.W., Kovacs,K., Pernicone,P.J., and Laws,E.R., Jr. (1996). p53 expression in pituitary adenomas and carcinomas: correlation with invasiveness and tumor growth fractions. Neurosurgery *38*, 763-770.

Theile,M., Seitz,S., Arnold,W., Jandrig,B., Frege,R. , Schlag,P.M., Haensch,W., Guski,H., Winzer,K.J., Barrett,J.C., and Scherneck,S. (1996). A defined chromosome 6q fragment (at D6S310) harbors a putative tumor suppressor gene for breast cancer. Oncogene *13*, 677-685.

Theodoropoulou,M., Barzon,L., Arzberger,T., Grübler,Y. Schaaf,S., Losa,M., Fallo,F., Ciminale,V., Stalla,G.K., and Pagotto,U. Variable menin expression in sporadic and familiar MEN1 pituitary adenomas as determined by immunohistochemistry. *Manuscript in preparation.*

Therrien,M. and Drouin,J. (1991). Pituitary pro-opiomelanocortin gene expression requires synergistic interactions of several regulatory elements. Mol. Cell Biol. *11*, 3492-3503.

Thrash-Bingham,C.A., Salazar,H., Freed,J.J., Greenberg,R.E., and Tartof,K.D. (1995). Genomic alterations and instabilities in renal cell carcinomas and their relationship to tumor pathology. Cancer Res. *55*, 6189-6195.

Toliat,M.R., Berger,W., Ropers,H.H., Neuhaus,P., and Wiedenmann,B. (1997). Mutations in the MEN I gene in sporadic neuroendocrine tumours of gastroenteropancreatic system. Lancet *350*, 1223.

Tomura,H., Okajima,F., Akbar,M., Abdul,M.M., Sho,K., and Kondo,Y. (1994). Transfected human somatostatin receptor type 2, SSTR2, not only inhibits adenylate cyclase but also stimulates phospholipase C and Ca2+ mobilization. Biochem. Biophys. Res. Commun. *200*, 986-992.

Tong,W. and Pollard,J.W. (2001). Genetic evidence for the interactions of cyclin D1 and p27(Kip1) in mice. Mol. Cell Biol. *21*, 1319-1328.

Tran,P., Zhang,X.K., Salbert,G., Hermann,T., Lehmann,J.M., and Pfahl,M. (1992). COUP orphan receptors are negative regulators of retinoic acid response pathways. Mol. Cell Biol. *12*, 4666-4676.

Tsai,S.Y. and Tsai,M.J. (1997). Chick ovalbumin upstream promoter-transcription factors (COUP-TFs): coming of age. Endocr. Rev. *18* , 229-240.

Varrault,A., Bilanges,B., Mackay,D.J., Basyuk,E., Ahr,B., Fernandez,C., Robinson,D.O., Bockaert,J., and Journot,L. (2001). Characterization of the methylation-sensitive promoter of the imprinted ZAC gene supports its role in transient neonatal diabetes mellitus . J. Biol. Chem. *276*, 18653-18656.

Varrault,A., Ciani,E., Apiou,F., Bilanges,B., Hoffmann,A., Pantaloni,C., Bockaert,J., Spengler,D., and Journot,L. (1998). hZAC encodes a zinc finger protein with antiproliferative properties and maps to a chromosomal region frequently lost in cancer. Proc. Natl. Acad. Sci. U. S. A *95*, 8835-8840.

Wang,L.H., Ing,N.H., Tsai,S.Y., O' Malley,B.W., and Tsai,M.J. (1991). The COUP-TFs compose a family of functionally related transcription factors. Gene Expr. *1*, 207-216.

Wang,L.H., Tsai,S.Y., Cook,R.G., Beattie,W.G., Tsai,M.J., and O' Malley,B.W. (1989). COUP transcription factor is a member of the steroid receptor superfamily. Nature *340*, 163-166.

Wang,Z., Yu,R., and Melmed,S. (2001). Mice lacking pituitary tumor transforming gene show testicular and splenic hypoplasia, thymic hyperplasia, thrombocytopenia, aberrant cell cycle progression, and premature centromere division. Mol. Endocrinol. *15*, 1870-1879.

Wenbin,C., Asai,A., Teramoto,A., Sanno,N., and Kirino,T. (1999). Mutations of the MEN1 tumor suppressor gene in sporadic pituitary tumors. Cancer Lett. *142*, 43-47.

Winkelmann,J., Pagotto,U., Theodoropoulou,M., Tatsch,K., Saeger,W., Müller,A., Arzberger,T., Schaaf,L., Schumann,E. M., Trenkwalder,C., Stalla,G.K. (2001). Retention of dopamine 2 receptor mRNA and absence of the protein in craniospinal and extracranial metastasis of a malignant prolactinoma: a case report. Eur.J.Endocrinol. *Manuscript in press.*

White,B.A. and Bancroft,F.C. (1983). Epidermal growth factor and thyrotropin-releasing hormone interact synergistically with calcium to regulate prolactin mRNA levels. J. Biol. Chem. *258*, 4618-4622.

Woloschak,M., Yu,A., Xiao,J., and Post,K.D. (1996). Frequent loss of the P16INK4a gene product in human pituitary tumors. Cancer Res. *56*, 2493-2496.

Xu,Y.H., Richert,N., Ito,S., Merlino,G.T., and Pastan,I. (1984). Characterization of epidermal growth factor receptor gene expression in malignant and normal human cell lines. Proc. Natl. Acad. Sci. U. S. A *81*, 7308-7312.

Zafar,M., Ezzat,S., Ramyar,L., Pan,N., Smyth,H.S., and Asa,S.L. (1995). Cell-specific expression of estrogen receptor in the human pituitary and its adenomas. J. Clin. Endocrinol. Metab *80*, 3621-3627.

Zhang,X., Horwitz,G.A., Heaney,A.P., Nakashima,M., Prezant,T.R., Bronstein,M.D., and Melmed,S. (1999a). Pituitary tumor transforming gene (PTTG) expression in pituitary adenomas. J. Clin. Endocrinol. Metab *84*, 761-767.

Zhang,X., Horwitz,G.A., Prezant,T.R., Valentini,A., Nakashima,M., Bronstein,M.D., and Melmed,S. (1999b). Structure, expression, and function of human pituitary tumor- transforming gene (PTTG). Mol. Endocrinol. *13*, 156-166.

Zhu,J., Leon,S.P., Beggs,A.H., Busque,L., Gilliland,D.G., and Black,P.M. (1994). Human pituitary adenomas show no loss of heterozygosity at the retinoblastoma gene locus. J. Clin. Endocrinol. Metab *78*, 922-927.

Zhuang,Z., Ezzat,S.Z., Vortmeyer,A.O., Weil,R., Oldfield,E.H., Park,W.S., Pack,S., Huang,S., Agarwal,S.K., Guru,S.C., Manickam,P., Debelenko,L.V., Kester,M.B., Olufemi,S.E. , Heppner,C., Crabtree,J.S., Burns,A.L., Spiegel,A.M., Marx,S.J., Chandrasekharappa,S.C., Collins,F.S., Emmert-Buck,M.R., Liotta,L.A., Asa,S.L., and Lubensky,I.A. (1997b). Mutations of the MEN1 tumor suppressor gene in pituitary tumors. Cancer Res. *57*, 5446-5451.

Zhuang,Z., Vortmeyer,A.O., Pack,S., Huang,S., Pham,T.A., Wang,C., Park,W.S., Agarwal,S.K., Debelenko,L.V., Kester,M., Guru,S.C., Manickam,P., Olufemi,S.E., Yu,F., Heppner,C., Crabtree,J.S., Skarulis,M.C., Venzon,D.J., Emmert-Buck,M.R., Spiegel,A.M., Chandrasekharappa,S.C., Collins,F.S., Burns,A.L., Marx,S.J., Lubensky,I.A. (1997a). Somatic mutations of the MEN1 tumor suppressor gene in sporadic gastrinomas and insulinomas. Cancer Res. *57*, 4682-4686.

Zou,H., McGarry,T.J., Bernal,T., and Kirschner,M.W. (1999). Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. Science *285*, 418-422.

Acknowledgements

This work was done in Max Planck Institute of Psychiatry (Director Prof. Dr. Dr. F. Holsboer), in the laboratory of Prof. Dr. G.K. Stalla, under the supervision of Dr. U.Pagotto, Dr. M.Paez-Pereda and Dr. U.Renner

I would like to thank:

Prof. Dr. Dr. Holsboer for giving me the opportunity to work in this Institute.

The Head of our group, Prof. Dr. Günter K. Stalla, for giving me the possibility to make my Ph.D. work in his lab. I thank him for offering me the great chance to enter in the fascinating world of pituitary adenomas. His expertise in the field largely contributed in the interpretation and formulation of the results presented in this thesis. He always provided me with motivation, and I thank him for his never-ending interest on my work.

Dr Ludwig Schaaf, for his support during my first two years in the lab.

My main supervisor, Dr. Uberto Pagotto, for giving me the opportunity to work in the fascinating projects of ZAC and Menin. I thank Uberto for following every step of this work, even when circumstances forced him to be far away from this lab. I thank him for the continuous encouragement and the support in the many difficult moments that work in the lab harbors. I am grateful for his boundless and quite infectious enthusiasm, which made working in the lab a pleasant and something-to-look-forward-to experience.

Dr. Marcelo Paez-Pereda, supervisor of the project for COUP-TFI, who entrusted me with a part of his project on retinoic acid and let me to witness and take care of the birth of a fascinating project. I thank Marcelo for being ready with his deep knowledge and scientific expertise to provide an advise, and many times a solution, to the small and big problems in the theory and practice of science.

Dr. Ulrich Renner, for always being eager to help me in the everyday problems of the lab and to provide a valuable advise.

Johanna Stalla, whose skillful technical expertise was a valuable help.

Yvonne Gruebler, for introducing me to many of the techniques I now know and in the general lab practice. This work wouldn't be completed without her large technical expertise and her will to help me out in the difficulties.

Dr Thomas Arzberger for introducing me to immunohistochemical techniques and for his valuable suggestions in the evaluation of in situ hybridization and immunohistochemistry.

The other people in the lab, my dearest colleagues, for always providing a friendly atmosphere, interesting comments and constructive discussions.

Dr. A. Yassouridis, whose statistical expertise made evident the significance of some of my results, and the neurosurgeons from several Neurosurgical departments in Germany for providing tumoral samples. I am also indebted to Dr. M. Losa (Institute San Raffaele, Milan, Italy) for providing an enormous amount of pituitary tumors and for generously giving thirteen rare and quite valuable cases of silent corticotrophinomas.

Dr. Maria Sasvari (Institute of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Budapest, Hungary), who first introduced me into the laboratory and whose care and enthusiasm inspired me to continue in academic career, and my lecturer in Biochemistry and tutor during my Master degree studies, Dr. Istvan Venekei (Department of Biochemistry, Eötvös Loránd University, Budapest, Hungary) for strongly advising me to enroll in a Ph.D. program!

My friends Ilke Inceoglu, Magdalena Sauvage, Masha Tichomirova and Vania Kapetaniou for constantly giving me courage during the difficult moments

My parents Anna Halatsi and Socrates Theodoropoulos for always enveloping me with their encouragement and support and for having led with certainty my first steps towards science.

CURRICULUM VITAE

Marily Theodoropoulou

Born at 1st of June 1974, in Athens, Greece

Education

1992	High school, 'Douka Educational Institutes', Athens, Greece
1992-1995	Biology-Basic studies, Faculty of Science, Eötvös Loránd University Budapest, Hungary
1995-1997	M.Sc. studies, Institute of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Budapest, Hungary
1997	M.Sc. in Biology (Note 4; best note: 5), Faculty of Science, Eötvös Loránd University, Budapest
1997-2001	Ph.D. studies, Neuroendocrinology Group, Max Planck Institute of Psychiatry, Munich, Germany

Dissertations

1997	*'Prenatal diagnosis of congenital adrenal hyperplasia due to 21-hydroxylase deficiency by allele specific PCR'* ,Master thesis, Eötvös Loránd University, Budapest

Publications

1. J. Trojan, M.Theodoropoulou, K.H. Usadel, G.K. Stalla, and L. Schaaf. Modulation of human thyrotropin oligosaccharide structures – enhanced proportion of sialylated and terminally galactosylated serum thyrotropin isoforms in subclinical and overt primary hypothyroidism; *J Endocrinol 158, 359-365; 1998.*

2. L. Schaaf, M.Theodoropoulou, A. Gregori, A. Leiprecht, J. Trojan, J. Klostermeier, G.K. Stalla Thyrotropin-releasing hormone time-dependently influences thyrotropin microheterogeneity--an in vivo study in euthyroidism. *J Endocrinol 166, 137-43; 2000.*

3. M. Theodoropoulou, T. Arzberger, Y. Gruebler, Z. Korali, P. Mortini, W. Joba, A.E. Heufelder, G.K. Stalla, and L. Schaaf. Thyrotropin receptor protein expression in normal and adenomatous human pituitary. *J Endocrinol 167, 7-13; 2000*.

4. U. Pagotto, T. Arzberger, M. Theodoropoulou, Y. Grubler, C. Pantaloni, W. Saeger, M. Losa, L. Journot, G.K. Stalla, D. Spengler. The expression of the antiproliferative gene ZAC is lost or highly reduced in nonfunctioning pituitary adenomas. *Cancer Res. 60:6794-9; 2000*.

5. U. Pagotto, G. Marsicano, F. Fezza, M. Theodoropoulou, Y. Grübler, J. Stalla, T. Arzberger, A. Milone, M. Losa, V. Di Marzo, B. Lutz, G.K. Stalla. Normal human pituitary gland and pituitary adenomas express cannabinoid receptor type 1 and synthesize endogenous cannabinoids. First evidence for a direct role of cannabinoids on hormone modulation at the human pituitary level. *J.Clin.Endocrinol.Metabol. 86:2687-96; 2001.*

6. M. Theodoropoulou, C. Barta, M. Szoke, A. Guttman, M. Staub, T. Niederland, J. Solyom, G. Fekete, M. Sasvari-Szekely. Prenatal diagnosis of steroid 21-hydroxylase deficiency by allele-specific amplification. *Fetal Diagn. Ther. 16:237-40; 2001*.

7. M. Paez-Pereda, D. Kovalovsky, U. Hopfner, M. Theodoropoulou, U. Pagotto, E. Uhl, M. Losa, J. Stalla, Y. Grubler, C. Missale, E. Arzt, G.K. Stalla. Retinoic acid prevents experimental Cushing syndrome. *J. Clin. Invest. 108(8):1123-31; 2001.*

8. J. Winkelmann, U. Pagotto, M. Theodoropoulou, K. Tatsch, W. Saeger, A. Müller, T. Arzberger, L. Schaaf, E.M. Schumann, C. Trenkwalder, G.K. Stalla. Retention of dopamine 2 receptor mRNA and absence of the protein in craniospinal and extracranial metastasis of a malignant prolactinoma: a case report. *Eur.J.Endocrinol. 146: 81-88; 2001.*

9. T. Ferro, D.M. D' Agostino, L. BarzonM. Theodoropoulou, A. Rosato, G. Esposito, I. Cavallari, C. Pasquali, M. Boscaro, U. Pagotto, L. Chieco-Bianchi, F. Fallo and V. Ciminale. In situ analysis of Menin expression using a monoclonal antibody and laser scanning microscopy. *Manuscript submitted.*

10. M. Theodoropoulou, L. Barzon, T. Arzberger, Y. Grübler, L. Schaaf, M. Losa, F. Fallo, V. Ciminale, G.K. Stalla, and U. Pagotto. Variable menin expression in sporadic and familiar MEN1 pituitary adenomas as determined by immunohistochemistry. *Manuscript in preparation.*

11. M. Theodoropoulou, T. Arzberger, Y. Grübler, M.L. Jaffrain-Rea, J. Schlegel, M. Losa, A. Gulino, G.K. Stalla, and U. Pagotto. Epidermal Growth Factor Receptor mRNA and protein is not limited to a particular subtype of pituitary adenomas. *Manuscript in preparation*

i